İSTANBUL TECHNICAL UNIVERSITY ★ EURASIA INSTITUTE OF EARTH SCIENCES

THE BACTERIAL AND ARCHAEAL BIODIVERSITY OF INSUYU CAVE

M.Sc. THESIS

Ezgi TOK

Department: Climate and Marine Sciences
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Ezgi Tok, a M.Sc. student of ITU Eurasia Institute of Earth Sciences with student ID 601131009, successfully defended the thesis entitled "THE BACTERIAL AND ARCHAEAL BIODIVERSITY OF INSUYU CAVE", prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 25 Kasım 2016
Date of Defense : 23 Aralık 2016

FOREWORD

Firstly, I want to thank my advisor Professor H. Nüzhet Dalfes (Istanbul Technical University) for giving me the opportunity to transform my passion, caving, into my profession. Actually, even before caving, i always knew that i have to be in nature. I knew that iam fastinated by the detailed simplicity of nature, and the whole dynamism beyond the solid face of the Earth. It was not just because of its poetical beauties of course, beyond this, because of the pure science behind those beauties. So, i hope that this thesis is only a beginning. But, for this first step, i really want to express my sincere gratitude to my advisor. I would also express my thanks to Assoc. Professor Alper T. Akarsubaşı for allowing me to perform the analyses which constitute the basis my Master'of this thesis in his laboratory, but also his endless support, guidance and patience. Special thanks goes to Halil Kurt for his technical guidance and Büşra Ahata for her incredible efforts in the laboratory, but beyond that, for her personal assistance in every stage of my project. Additionally, I want to thank Ezgi Baştürk, İsmail Can Hacıosmanoğlu and rest of the lab-team for their precious support and all those cheerful moments. At the last, thanks mom, braveness, it is the most precious gift you gave me.

Kasım 2016 Ezgi Tok

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ABBREVATIONS

PCR : Polymerase Chain Reaction

QPCR : Quantitative Real-Time Polymerase Chain Reaction

EPS : Extracellular Polymeric Substance

HRM : High Resolution Melting Curve

AGE : Agarose Gel Electrophoresis

NGS : Next Generation Sequencing

DNA : Deoxyribonucleic acid

16S rDNA : 16S Ribosomal Deoxyribonucleic acid

RNA : Ribonucleic acid

16S rRNA genes : 16S Ribosomal Ribonucleic acid genes

dNTP : Deoxyribonucleotide triphosphate

Ct : Cycle threshold

OTU : Operational taxonomic unit

TAE : Tris-Acetate-EDTA

Tm : Melting Temperature

UPGMA : Unweighted Pair Group Method with Arithmetic Mean

V : Volts

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THE BACTERIAL AND ARCHAEAL BIODIVERSITY OF INSUYU CAVE

SUMMARY

Caves are one of the unique habitats to see interaction between rock and microbes. In the aspect of microbiological researches, caves are unique environments in order to not only the limitations on exposing to exterior environmental conditions but also the natural isolation for disturbants such as other living creatures.

In these harsh environments, Proteobacteria, Actinobacteria, Chlorobi/Bacteriodetes, Chloroflexi, Deltaproteobacteria, Acidobacteria, Nitrospirae, Actinobacteria and Betaproteobacteria are the most common and diverse group of bacteria because of their adaptation ability.

In this study, we conducted cultivation-independent 16S rDNA surveys on the bacterial and archaeal community of Insuyu Cave, Burdur, TURKEY. The complexity of microbial communities has been presented in samples through PCR amplification of the 16S ribosomal RNA (16S rRNA) gene sequence, high resolution melting curve (HRM) and next generation sequence (NGS) based on 16S rRNA metagenomics from a variety of cave samples to identify bacterial and archaeal community profile of Insuyu Cave.

A total of nine known bacterial class were found within the context of this research (Figure 3.4). The most prevalent class is alphaproteobacteria with 89,23% of the total bacteria found grouping into four order, with the most abundant ones: rhizobiales (88,85%) and sphingomonadales (0,21%). The followings, ordered by abundancy, are actinobacteria (3,94%), bacilli (2,92%), gammaproteobacteria (1,69%) and betaproteobacteria (0,74%).

Also, a total of fourteen genera from ten order were identified in this study (Figure 3.5). The most dominant is methylobacterium with 88,83%, from the class of rhizobiales (88,85%). The followings are propionibacterium (3,58%), dolosigranulum (1,69%), streptococcus (1,10%) and pseudomonas (1,13%).

On the other hand, no Archaeal species has been detected at the result of NGS analysis while some archaeal nucleic acid had been marked as a result of PCR analysis. The reasons of this situation has been investigated in the section of discussion.

İNSUYU MAĞARASI'NIN BAKTERİYEL VE ARKEAL BİYOÇEŞİTLİLİĞİ

ÖZET

Mağara, yüzeyin altında, alacakaranlık bölgesinin ötesine uzanan ve insanlara açık olan herhangi bir doğal boşluk olarak tanımlanır (Ford, 2007; Northup and Lavoie, 2001; Hill and Forti 1997; Gillieson 1996). Mağaralar, çevresel koşullara, içinde geliştiği kayacın türüne veya bölgesel tektonizmanın kuvvetleri gibi jeolojik faktörlere bağlı olarak birçok çeşitte şekillendirilebilir. Hiçbiri resmen kabul edilmese de, mağaraların sınıflandırılmasında yaygın olarak kullanılan bazı kriterler, mağaranın oluşum mekanizması ve oluşumun meydana geldiği sürecin anakaya oluşumunun hangi basmağına denk geldiğidir. Speleogenesis süreci esas alınarak belirlenmiş sekiz temel doğal mağara grubu vardır. Bunlardan altısının da, "Denizel mağaralar", "Talus mağaraları", "Buzul mağaraları", "Akarsu mağaraları", "Çatlak mağaraları" ve "Ağsı mağaralar", dünyanın dört bir yanında pek çok örneği bulunurken özellikle ikisi, "Volkanik mağaralar" ve "Karstik mağaralar" olarak adlandırılanlar, dünya çapında en yaygın görülenlerdir (Palmer, 2007).

Mağaralar, düşük sıcaklık, yüksek nem, daimi karanlık, organik madde girdisinin olmaması gibi bir takım çevresel koşullar sebebiyle çoğunlukla ekstrem ortamlar olarak tanımlanır. Bu ekstrem koşullar sebebiyle, habitattaki mikroorganizma popülasyonu genellikle, enerji kaynağı olarak indirgenmiş inorganik bileşikleri kullanabilen, kemolitotroflar başta olmak üzere kemoototroflar tarafından oluşturulmaktadır. Kemolitotroflar, ATP sentezi sırasında oksidasyondan faydalanmakta ve bu esnada, bir kısım mineral bileşiklerin redoks tepkimeleri ile oluşmasını sağlamaktadırlar. Ayrıca, bu mikroorganizmaların zor koşullar altında yaşayabilmesini sağlayan, örneğin yüzeye tutunmak için hücre dışına polisakarit bazlı bir madde salgılamaları gibi, bir takım metabolik faaliyetleri de bu ortamlarda mineralizasyon süreçlerini desteklemektedir.

Şu ana kadar, dünyada yürütülmüş çalışmaların çoğu son yirmi yıla ait olmakla beraber, 1990 öncesinde yalnızca birkaç çalışmayla sınırlı olan bu alan son on yılda hızla popüler araştırma alanlarından biri olmuştur. Mikrobiyolojik araştırmalar açısından bakıldığında, yüzey atmosfer koşullarına ve diğer canlı türlerinin yarattığı dalgalanmalara karşı korunaklı olan mağaralar ekstrem koşullarda canlılık, mikrobiyallerin yöneldiği alternatif enerji türleri, popülasyonlar arasındaki ilişki gibi konuların incelenmesi için benzersiz ortamlardır.

Mağara mikrobiyolojisi çalışmalarının çoğu, ikincil mağara birikimlerinde, yani speleotemlerde yaşayan bakteri toplulukları üzerinde yapılmıştır. Proteobakteriler, adaptasyon yetenekleri nedeniyle en yaygın ve çeşitli bakteri grubudur. Ayrıca, hipojenik mağaralarda Deltaproteobakteriler, Asidobakteriler, Nitrospiler, Aktinobakteriler ve Betaproteobakteriler baskın grup oluştururlar.Çoğunlukla popülasyon kükürt oksitleyenler, nitrit-nitrat döngüsünde rol oynayanlar, demir oksitleyenler ve hidrojen oksitleyenler üzerine yoğunlaşmaktadır.

Bu çalışmada, Burdur İl merkezine yakın bir doğal karstik mağara olan İnsuyu mağarasından elde edilen speleotemlerden, bu speleotemlerin oluşumu ile ilişkili

olabilecek, arkea ve bakteri kolonileri izole edilmesi hedeflenmiş ve çeşitli yöntemler kullanılarak tür tespiti yapılmıştır.

Deney için, alınan 18 örnekten mineralojik açıdan diğerlerinden farklı olan 11'i seçildi. DNA, üretici protokolündeki değişiklikler yapılarak PowerSoil® DNA İzolasyon Kiti (MO BIO Laboratories Inc., CA) tarafından izole edildi. İlk denemelerde, agaroz jel elektroforezi sonuçları DNA ekstraksiyonu sırasında olası bir inhibisyonu işaret ettiğinden, bunun sebebi araştırıldı ve XRF analizi ile inhibisyon faktörü Ca ++ olarak belirlendi. DNA ekstraksiyonunun verimini arttırmak için, alternatif katyonik polimer, poli-deoksiinosinik-deoksisitidik asit sodyum tuzu, kullanıldı.

DNA ekstraksiyonundan sonra, her örnek agaroz jelde yürütülerek analiz edildi. Jeller, 0.5 ug / mL etidyum bromür içeren 1XTAE tamponu içerisinde % 1 (ağ / hac) agaroz kullanılarak hazırlandı. 4ul DNA örnekleri, 2x jel yükleme tamponu ile karıştırıldı. Elektroforez 10V / cm'de gerçekleştirildi ve jel, jel görüntüleme sistemi (Gel Doc, BIORAD, ABD) kullanılarak UV altında görselleştirildi. Örnekler, kullanılıncaya kadar -20 ° C'de saklandı.

Mikrobiyal topluluklar, ilk önce ekstraksiyon sonrasında yorum yapabilmek için yeterli 16S rRNA geni elde edilememesi sebebiyle, amplifiye edildi. 16S rRNA genine dayalı PCR (Polimeraz Zincir Reaksiyonu) metodolojisi ile izlendi. Mikrobiyal rDNA'ları çoğaltmak için Nested PCR yaklaşımı kullanıldı.

Daha sonra, PCR ürünleri, 1X TAE tamponu (40 mM Tris bazı, 20 mM asetik asit, 1 mM 0.5 M etilendiamintetraasetik asit, pH 8.0) içinde etidyum bromür ile boyandıktan sonra% 1,5 agaroz jel üzerinde ayrıldı. AGE 100 V cm-1'de 30 dakika süreyle gerçekleştirildi ve ürünler trans-UV (BIORAD, USA) kullanılarak izlendi. Bu işlem sonucunda bakteriyel ve arkeal 16S rRNA genlerinin tüm örneklerde mevcut olduğunu doğrulandı.

Ardından mağara tortullarındaki arkeal ve bakteriyel çeşitlilik, HRM kullanarak ışıma grafiklerindeki benzerliklere göre karşılaştırılarak incelendi. Mikrobiyal rDNA'ları çoğaltmak için Nested qPCR yaklaşımı kullanılmıştır (Kolukırık ve diğerleri, 2011).

HRM analizi sonuçlarından yola çıkılarak, mikrobiyal topluluk profili ile örneklerin HRM profilleri arasındaki benzerlikler koröle edilerek dendrogram grafiği çizildi.

Bu grafiğe göre, 11 numuneden, mağara ekosistemindeki farklı toplulukların her birini temsil etmesi açısından birbirlerine benzerlik açısından ortalama bir değer gösteren 7'si, 16S rRNA Metagenomik Dizilim için seçildi.

Ardından, seçilen I3, I6, I13, I16, I17 ve I18 örnekleri, 16S rRNA Metagenomik'e dayalı gen dizileme yöntemi ile analiz edildi. Diziler, SILVA veri tabanı kullanılarak en yakın sınıf / cins ile gruplandırıldı.

Bu araştırma bağlamında toplam dokuz bilinen bakteri sınıfı bulunmuştur. En yaygın sınıf alfaproteobakteri olup, temel olarak dört grupta toplanacak şekilde toplam bakterilerin % 89,23'ünü oluşturur. Bunlardan en yoğun populasyonlar ise rhizobiales (% 88,85) ve sfingomonadales (% 0,21)'dir. Aktinobakteriler (% 3,94), basil (% 2,92), gammaproteobakteriler (% 1,69) ve betaproteobakteriler (% 0,74)'de bu grubu takip etmektedir.

Ayrıca, bu çalışmada on sınıftan toplam on dört cins tespit edilmiştir. En baskın olanı metilbacterium'dur (% 88,83), rhizobiales sınıfından (% 88,85). Propionibakteri (% 3,58), dolosigranulum (% 1,69), streptokok (% 1,10) ve psödomonas (% 1,13) takip etmektedir.

Bununla birlikte, PCR analizi sonuçlarında tespit edilmesine ragmen, NGS analizlerinde herhangi bir arkea grubu gözlemlenmemiştir. Bu durumun olası nedenlerine ise tartışma kısmında yer verilmiştir.

1. INTRODUCTION

1.1 Aim

Although caves are the extreme ecosystems, microbial communities dwelling caves have a profile as wide diversity. Such studies will continue to provide the significant information which maybe used for amelioration of the alternative energy sources, the novel technologies on medical or the better method for wastewater treatment, and to illuminate us about the biological adaptations on the extreme and dark conditions, the influence of geochemically extreme conditions on microbial diversity, the unique microorganisms and metabolites, the microbial chemolithotrophic/ heterotrophic ecosystem processes and the role of microorganisms in biomineralization and the microbial interactions with the mineral surfaces.

In this study, we describe the isolation and phlogenetic identification procedure of archaea and bacteria species derived from speleothems collected Insuyu cave, a karstic cave located near the center of Burdur Province, Turkey.

Finally, we discuss the possible connections between the communities of bacteria and archaea isolated from different locations in cave and the environment where they settled on. Also, the difficulties at the monitoring of the microbial community isolated from environmental samples have been discussed.

1.2 General Information about Karstic Caves

1.2.1 Cave: Definition and classification

A “cave” is defined as any natural space below the surface, providing that extends beyond the twilight zone, and that is accessible to humans (Ford, 2007; Northup and Lavoie, 2001; Hill and Forti 1997; Gillieson 1996).

Caves can be shaped in many varieties depending on the environmental conditions and the geological factors such as the type of host-rock or the forces of the regional tectonism. Although none of them are formally accepted, there are some criteria used commonly for the categorization of caves which are the origin of the cave, the type of the host-rock and the stage

of the rock forming processes on which the speleogenesis occurs (Lee et al., 2012; Palmer, 2007; Northup and Lavoie, 2001). On the other hand, any of these criteria could not be considered as a certain classification since the complexity of the speleogenesis.

There are eight basic groups of natural caves which are categorized by the process of the speleogenesis or, in other words, the origin of the cave. While six of them, the ones named as "Wave-cut caves" "Talus caves" "Glacier caves" "Stream-cut caves" "Crevice caves" and "Framework caves", also have many examples around the world, two of them, the groups called as "Volcanic caves" and "Karstic caves", are the widest ones of them (Palmer, 2007). The mechanisms of speleogenesis can be driven by the chemical, physical or both of these processes according to the type of the host-rock and the environmental conditions. Depending on this criterion, caves are also classified as either primary or secondary by considering that the stage of bedrock formation on which the speleogenesis happens. Those which is formed at the same time as the host-rock is called as primary, otherwise, if the speleogenesis process happens after the completion of the host-rock forming process, then the cave is called as secondary (Palmer, 2007).

1.2.2 Speleogenesis of caves

Caves may be formed by chemical and physical forces, or sometimes both. "Wave-cut caves", also known as "Littoral caves", develop on the coastlines at which the waves converge and amplify their erosive forces. These caves are grouped as secondary, as that the mechanical force of waves erodes the host-rock which is generally limestone and sandstone in addition to mudstone and basalt. Similarly, "Talus caves" which are comprised of voids between fallen boulders, "Crevice caves" which constitute enlarged fissure system by erosional forces on the fractured insoluble rocks such as granites, even tallus, "Stream-cut caves" which belong to the sub-group of crevice caves, "Glacier caves" which are formed by partially melting of ice by hot water streams through fissures in the surface or edges of glaciers are considered as secondary cause of their genesis (Palmer, 2007; Northup and Lavoie, 2001). However, "Framework caves" are primary caves within the structure produced by accumulation of sediments. Also, the group known as "volcanic caves" or "lava tubes" are primary caves. The mechanism is unique to volcanic caves such as lava tubes, explained as consequence of that the inner part of molten lava erupting to surface remains to flow while the outer layer solidifies cause of cooling just after the eruption (Lee et al., 2012; Engel, 2010). As molten lava flows out of a volcano, the surface lava cools more quickly and solidifies. When the eruption stops, the rapidly owing lava may drain, leaving an empty tubular conduit behind.

The widest group of caves is called as “solution caves” extending in soluble bedrocks such as mudstone, sandstone, gypsum, hallides but mainly at the karstic terrains which is covering 15-20 % of earth’s surface. The term “Karstic caves” is used for these special type of solution caves which are the most known secondary caves observed karstic terrains. Dissolution can be both epigenic and hypogenic processes due to the direction of corrosive effect of water which are on or through the rock (Engel, 2010; Ford, 2007). In case of epigenic cave formation, dissolution starts from the upper part of the stratum and continues downward within the corrosive action of meteoric water (e.g. dripping water) due to the infiltration. The dominance of hot and wet conditions inducing the high precipitation and the activity of microbes in an area has the main role on the dissolution process, especially karstic terrains, by releasing carbonic acid derived from the reaction of the carbon dioxide absorbed from the soil and the surface water. As the acidic water reaches the water table, it stays in contact with the limestone and dissolves more calcium carbonate (Palmer, 2007; Gillieson, 1996). As the water reaches the cave, carbon dioxide degasses into the cave air, which allows the formation of calcium carbonate speleothems such as stalactites and stalagmites (Northup and Lavoie, 2001; Forti and Hill, 1997). However, the surface water has insignificant to zero effect on the formation process of hypogenic caves arising with the fluids moving through the subsurface. This mechanism depends on the interaction with the groundwater table of the cavity. The most known agent to form hypogenic caves is sulphuric acid originating from both biotic and abiotic processes. Sulphuric acid is thought to form by volatilization of hydrogen sulphur from the groundwater rising along until it meets the cave atmosphere placed generally in the vadose zone and its subsequent autoxidation in the moist cave walls by causing dissolution of limestone (Klimchouk, 2009; Northup and Lavoie, 2001; Hill, 1990). The sulphur-oxidizing bacteria have also been suggested to generate sulphuric acid as a by-product in caves systems (Macalady et al., 2006; Engel et al., 2004; Hose et al., 2000) thus potentially contributing to cave development. In addition to dissolution, erosion is the other mechanism contributing genesis of caves. It is possible that erosion process can be seen with dissolution process in the many cases of karstic caves.

1.2.3 The typical niches at the karstic caves

Caves are generally considered extreme environments due to the absolute darkness past the twilight zones which results in inadequate photosynthetic activity and nutrient-limited environment to sustain the viability of organisms (Jain et al., 2010; Baskar et al., 2009; Northup and Lavoie, 2001). Depending on these conditions, low productivity and biomass are the wide-

spread results. Physical parameters are mostly stable, characterized by high humidity (95-100%) and constant temperature. (Tomczyk-Zak and Zielenkiewicz, 2015; Pasic et al., 2009).

According to the susceptibility from the surface in the meaning of the environmental conditions, caves can be divided into four habitation zones, which are the entrance zone that is exposed to sunlight and changes on surface temperature which are the conditions extending occupation of terrestrial vegetation; the twilight zone characterized with scarce in light and minor changes in temperature depending on outside conditions; the transition zone susceptible to surface conditions excluding light which is extremely constraining on the plant life and the dark zone characterized by complete darkness and constant temperature forcing the dark zone to be highly oligotrophic due to the limited allochthonous energy sources or chemoautolithotrophic activities to support the ecosystem. In contrast, the entrance and the twilight zones contain of higher biodiversity since the presence of sunlight provides energy to the region (Hobbs and Culver, 2009).

Food sources in subterranean habitats are constrained by the lack of photosynthesis behind the entrance zone, thus herbivore dependent primary productivity shows no existence usually at caves past of this zone. Some shallow caves are supported by penetrating plant roots but most caves depend on allochthonous sources of organic material. This allochthonous material is produced in the surface and reaches the cave through the entrance, the epikarst (zone in the upper few meters of the bedrock above the cave and characterized by enlarged fissures and pores) and sinkholes as dissolved or particulate organic matter (Hobbs and Culver, 2009). Water infiltrating through the epikarst or sourced by underground streams and air flow came from entrance can also bring invertebrates, bacteria and fungi beside of death plants that serve as food for other organisms. Another major source of organic matter is provided by the fecal material of crickets, birds, rats, raccoons and bats. However, communities that live on fecal material are concentrated within a few hundred meters of the cave entrance (Hobbs and Culver, 2009). After entrance zone, the material obtained from infiltrated water and underground streams are still effected on the energy budget of the zone but the chemolitotrophic activity takes place as the main element of the energy cycle at rest of the cave which became a real oligotrophic system.

Cave inhabitants or troglofauna/flora -stygofauna/flora are grouped into three categories which are trogloxenes, troglophiles and troglobites according to their life-history that embrace their preference about the zone of the cave to residence, besides their duration of dwelling. Trogloxenes are visitor organisms that residence in the entrance or twilight zone and use the

cave as a shelter. In addition to being a shelter, also, bats, the most studied one of trogloxenes, use caves as nursery roost. Troglophiles, which are also incomer organisms living in the entrance or twilight zone, prefers to stand their entire life in the cave within the capability of surviving outside (Lee et al., 2012; Hobbs and Culver, 2009; Howarth, 1980). Troglobites are the cave adapted residents which dwell at the dark zones permanently and the reliable minimal energy sources. These organisms have distinctive phenotypic features as reduced metabolism, disappeared pigment (depigmentation) and eye (anophtalmy) in addition to development of specialized sensory structures (Lee et al., 2012; Howarth, 1980). For instance, the most of the cave dwellers have longer palpusses than their outsider ancestors. For some cases, the sensory organs have replaced at the edge of their limb. Some examples of troglobites include blind fishes with the species name Amblyopsis *rosae* and Arachnocampa *luminosa* the glowing fungus gnat (Hobbs and Culver, 2009; Gatenby, 1960).

1.2.4 Bacterial and Archaeal profiles of karstic caves

The most of cave microbiology studies has been conducted on the bacterial communities inhabiting secondary cave deposits. Proteobacteria is the most common and diverse groups of bacteria because of their adaptation ability (Engel, 2010; Ettema and Andersson, 2009). Actinobacteria, Chlorobi/Bacteriodetes and Chloroflexi follow the group Proteobacteria. Deltaproteobacteria, Acidobacteria, Nitrospirae, Actinobacteria and Betaproteobacteria constitute of dominant group in hypogenic caves, specially. (Engel et al., 2010; Barton et al., 2007). Types of visible colonies of bacteria observed on rock surfaces could be grouped in three which are described as grey, yellow or white dots (Cuezva et al., 2009; Saiz-Jimenez et al., 2011; Saiz-Jimenez, 2012).

Also, there are some studies reporting the abundance of archaeal communities in caves (Northup et al., 2003; Gonzalezet al., 2006; Macalady et al., 2007; Spear et al., 2007; Chen et al., 2009). Referred to review of plenty studies, Crenarchaeota and Euryarchaeota are the dominant groups of Archaea in cave environment (Northup et al., 2003; Macalady et al., 2007; Spear et al., 2007). However, there is not enough evidence to make a comment on archaeal communities in the caves.

1.3 Literature

1.3.1 Historical background of the researches in karstic caves

Microbial life on earth had to evolve in an environment prior to photosynthesis when there was limited nitrogen and where the majority of organisms used minerals for sources of energy (Baskar et al., 2009). The prokaryotic form of life not only persists today but thrives and continues to evolve.

Anton van Leeuwenhoek (1632 – 1723) described the shape of "very little animacules" which are bacteria, yeast, and protozoa in 1675. After several years, Sergei Winogradsky (1845 – 1916) and Martinus Beijerinck (1851 – 1931) determined the role of bacteria on biochemical cycles of the planet and indicated to "Chemolitotrophy" as the term explaining the relation between soil/rock and the metabolic activity of microbes, in 1889. Subsequently, the researches on geomicrobiology field have accelerated by the support of many biologists such as Lorenz Hiltner (1862-1923) who proposed the term rhizosphere, a pioneer in microbial rhizosphere ecology (Barton and Northrup, 2011). The developments of geomicrobiology marked the beginning of a new era of natural interdisciplinary sciences composed by chemistry, biology and geology in addition to rhizosphere ecology, the biology of the root zone, which was just first one of them.

From early 70s, the microbe–mineral interactions at critical zones described as where rock meets life have taken the spotlight (Banks et al., 2009). At 1977, the discovery of deep-sea vent ecosystems has highlighted the fascinating nature of extreme environments. On the other hand, the microbial life of cave ecosystems could be point of interest just about at the twentieth century by the earliest publications which are Ove Arbo Høeg's written about microbes on the walls of Norwegian caves and Caumartin's gathering informations about cave microorganisms (Lee et al., 2012; Northup and Lavoie, 2001).

The beginning of the cave microbiology was based on culture-based methods and microscopy (Barton, 2007). While there are plenty of growth media for common microorganisms, to grow most of subsurface environmental microorganisms which in the group of extremophiles engages a respectable amount of laboratory experiments to stimulate their primary environment in growth media. It is assessed that only the one inch of soil has alive microbial cells more than the number of eukaryotic organisms living all over the world. Moreover, less than one percent of the cells can be monitored by this kind of laboratory-based experiments (Amann, 1995; Winogradsky, 1946). As far as cave ecosystems are concerned, this percentage could decline in

order to the struggle with design of the microbial growth media which is appropriate for microbial community composed dominantly by extremophiles that can thrive in physically or geochemically extreme conditions (Barton and Northrup, 2011; Barton, 2006). Therefore, to practice geomicrobiology study on cave environment affords invaluable opportunities for the observations on biological adaptations within the extreme and dark conditions in the meaning of how the geochemically extreme conditions of such environments can influence microbial diversity, the discovery of unique microorganisms and metabolites in addition to enlightenment of the obscurities about microbial chemolithotrophic/heterotrophic ecosystem processes, the role of microorganisms in biomineralization and the microbial interactions with the mineral surfaces they are associated with (Baskar et al., 2009).

Nevertheless, despite these motivations, studies of microorganisms which examine the microbial community profiles and the environmental factors adjusting the diversity in caves had limited with just a few experimental studies till recent. By the past two decades, extensive researches in cave environments have began to produce (Northup and Lavoie, 2001). Within the findings from one of the earliest researches about the chemoautotrophically sustained Movile Cave in Romania, it has been considered about the potential to study the use of alternative sources of energy in such environments (Kumaresan et al., 2014; Baskar et al., 2009; Sarbu et al., 1997; Sarbu et al. 1996). Subsequently, it has began to elucidate how the fungal activity, the archaeal activity and the chemolithoautotrophic bacterial activity mainly associated with sulfur oxidizing bacteria, sulfate reducing bacteria, calcifying bacteria, aerobic methane-oxidizing bacteria and nitrite-oxidizing bacteria can influence to geochemistry in the subsurface of the Earth (Kumaresan et al., 2014; Hathaway et al., 2014a; Banks et al., 2010; Engel et al., 2010; Canganella et al., 2007; Spilde et al., 2005; Canaveras et al., 2001; Jones, 2001).

1.3.2 Researches on bacterial and archaeal communities in karstic caves

The bacterial and archaeal community researches performed in cave environment can be grouped into three topics. These are biodivesity, biomineralization and extraterrestrial life researches.

Multiple studies have been approached on microbiodiversity in cave environments to date. Although these studies have mainly conducted in karstic caves, there are also examples carried out in lava caves as in the two studies documented by Hathaway et al. (2014a, 2014b) about on the identification of ammonia oxidation (amoA) and nitrogen fixation (nifH) communities colonizing in lava caves of Terceira, Azores in first paper in addition to comparising whole

bacterial diversity of these caves with the bacterial diversity in Hawai'ian Lava Cave Microbial Mats on the other one.

On the other hand, the biodiversity studies at karstic caves are more common and more comprehensive. For instance, Barton et al. (2004) have conducted the first non-cultured phylogenetic analysis of the bacterial community in Fairy Cave, Colorado, USA in 2004. Gonzalez et al. (2005) have investigated microbial proliferation on paleolithic paintings dating back to 15,000 years in Altamira Cave. On the other research, Pasic et al. (2010) have focused on understanding the distribution of microorganisms in cave environments within the context of their paper about microbial communities of a karstic cave in Slovenia. The light-reflecting microbial communities colonizing the walls were observed throughout the visited gallery of Pajsarjeva jama cave by this publication.

In addition to biodiversity researches, there are also plenty about biomineralization in caves. Secondary mineral deposits elderly considered as a purely inorganic pathway have been reevaluated if it has an organic origin also. By increasingly number, it is claimed that interactions of cave microorganisms and host rocks can lead to constructive and destructive processes of minerals and speleothems with mainly 38 described types, based on physico-chemical reactions (Barton et al., 2007; Melim et al. 2001).

The depositions of calcium carbonate, oxidized iron-manganese, oxidized ammonium and nitrate formation, released elemental sulphur and the disintegration of the host rock can be exemplified as the most familiar ones of the documented results of biogeochemical activity in these environments (Northup and Lavoie, 2001). Also, a speleothem arising from organic origin can be constituted more than one of these minerals regarding to the number of metabolic reactions in a particular microbial niche. Over 250 different minerals have been described in caves to date (Hill and Forti, 1997).

Owing to calcium carbonate speleothems are the most extensive type of secondary deposits among caves, a number of studies have done on the bacterial precipitation of calcium carbonate in vivo or in vitro (Baskar et al., 2014a; Rusznyák et al., 2012; Sanchez-Moral et al., 2012; Banks et al., 2010; Cacchio et al., 2004; Le M´etayer-Levrel et al., 1997). As an example, Baskar et al. (2014a) have identified biogenic evidences of secondary cave formations composed by calcium carbonate in the Mawmluh Cave, Meghalaya, India in 2011. Similarly, Melim et al. (2001) have indicated evidence for microbial involvement in secondary cave deposits at Hidden Cave, New Mexico. Also, the possible mechanism of calcium carbonate precipitation has been described by a many of researches. For example, it has suggested that microbial colonization is

essential for secondary mineralization in order to the high affinity for calcium ions of microbial biofilms (Canaveras et al., 2001). As another example, the study of bacterial calcium carbonate precipitation in cave environment on the samples collected from an unnamed cave in Kentucky, USA could be marked (Banks et al., 2010). The group has suggested that it is a detoxification mechanism to survive at the high concentration of calcium. Further, Baskar et al. (2014b) demonstrated that bacterial carbonate precipitation has two pathways which are active (photosynthesis, urea hydrolysis, sulfate reduction, and iron reduction) or passive pathways (nucleation sites) by the study of calcifying bacteria isolated from Sahastradhara Caves in Siwalik Himalaya, India.

As well as calcifying bacteria, the metabolic activity of iron and manganese oxidizing bacteria colonizing on ferromanganese deposits have been evidenced to contribute the secondary mineralization in cave environments (Pacton et al., 2013; Lozano and Rossi, 2012; Barton and Northup, 2007; Spilde et al., 2005; Peck, 1986). As an instance, Peck demonstrated that the metabolic precipitation mechanisms to enhance Fe- and/or Mn-oxide formation chemolithotrophically by the cultures of iron-oxidizing bacteria isolated from cave pools in Level Crevice Cave, Iowa in 1986. In the another study, Pacton et al. (2013) have showed that the Fe and Mn deposits in the Siberian stalactite has derived from microbial activity, by laboratory based experiments. Pacton's experiments constructed to monitor both abiotic and biotic environmental conditions have confirmed that the depositions in the same formation and appearance with ones observed on stalactite in cave only engender in the biotic system and showed that the Fe-oxide formations biologically induced by EPS (extracellular polymeric substances) form in a different morphology than the formations generated in the abiotic conditions. Lozano and Rossi (2012) have also reported that the stromatolites sampled from El Soplao Cave mainly exists in the areas containing relatively pure polymetallic Mn-rich oxides within the dendritic and laminar microfacies, while they spread limitedly in areas with significant detrital material. It has indicated that his extracellular precipitation induced by microbial metabolism provides an exceptional natural shield to stromatolites.

Besides, nitrate formation in caves has been likewise reported as a result of microbial activity. In 1990, Hess was the first who marked that saltpeter deposits (a diverse array of nitrate complexes) were related to microbial activities (Barton and Northup, 2007). He had proposed that nitrates are the evaporated residuals of outsider bacterial activity. Furthermore, there are phylogenetic studies proving nitrogen fixation, ammonia oxidation and nitrification mechanism occuring from both bacterial and archaeal origin in cave (Barton et al., 2004). Some researchers

have isolated bacterial and archaeal amoA genes in Movile Cave, a mine adit in Colorado in addition to that Nitrospira and Nitrobacter has identified on many studies (Barton and Northup, 2007). These findings can be considered as evidences for possible role of ammonia oxidation and oxidation of nitrite to nitrate, the first and the second step of nitrification, as an energy strategy for cave microorganisms. Nevertheless, even today there are doubts on relation between microorganisms and both denitrification and nitrite oxidation only identified by phylogenetic methods (Barton and Northup, 2007).

The other one of the main reactions detected in caves is reaction occurring between a variety of hydrogen sulphide and O/CaCO/HO by provoking the deposition of elemental sulphur and gypsum. There are a lot of sulphur spring throughout the world, but, spring at caves of the Villa Luz has a unique structure with the upstream engendering the necessary conditions for carbonate dissolution by oxygenation. The oxidation of H_2S releases sulphuric acid involving in the alteration reaction of limestone to gypsum (Hose et al., 2000). As another instance, Engel et al. (2004) have isolated that almost all of the sulfur-oxidizing bacteria which consume H_2S. The researchers have described the process as that the bacteria colonizing on carbonate surfaces produces sulfuric acid and sulfuric acid speleogenesis occurs by increasing local carbonate saturation. Furthermore, they have suggested that the metabolic activity of sulfur-oxidizing bacteria endures, even if partial pressure of oxygen is critically low for autoxidation, providing the extensive penetration zone to support speleogenesis at deeper strata.

Beside of the fields microbial diversity and biomineralization, there is also a novel research area emerged last decade. Although caves are unique ecosystems to some extent, it was suggested that caves can be considered as a counterpart for the studies carried out to detect and elucidate the life on other planets (Lee et al., 2012; Boston et al., 2001; Boston, 2000). For example, lava caves are considered as analogs to comprehend how to examine volcanic activity and lava caves on Mars to find evidence of life on Mars (Boston 2010; Boston et al. 2001, 1992)

Altough there are many significant studies undertaken microbial communities in various type of caves from different continents, researches on cave geomicrobiology and biomineralization are very scarce in Turkey. The researches conducted by Barış et al. (2012a; 2012b; 2010a; 2010b; 2010c; 2008a; 2008b; 2008c) and Güleçal et al. (2013; 2016a; 2016b) are the only examples of microbiological studies at cave environment in Turkey.

As the primary study on cave microbiology in Turkey, Barış et al. (2008a; 2008c) have investigated the bacterial flora of the dripstones of Yıldızkaya (Kivi) Cave. The similar study has also conducted by using different method, ARDRA (Amplified Ribosomal DNA

Restriction Analysis), by the group in 2012 (Barış et al., 2012b). The effects of bacteria on calcite formation in cave systems were subjected to the author's second study. The microbial colonies have isolated from the dripstones of limestone caves in Erzurum region and cultured. Calcite formation was observed on agar plates as a result of experiment (Barış et al., 2008b). Also, the biomineralization process has confirmed by the author's other studies performed at the same region (Barış et al., 2010b; 2010c). The third subject of the group is the bacterial metabolism in the oligotrophic cave environment. The study of the group has showed that the generation rate of some bacteria at the poor media condition is even higher than at the environment which is rich in organic matter, beside of the result that some bacteria can not colonize in normal media while they can do it in poor media (Barış et al., 2010a; 2012a).

As the second group, Güleçal and Temel (2013; 2016) have focused on Sulfur cycling and bacterial diversity in cave environment. The diversity of sulphur oxidizer bacteria and their metabolism in Kaklık Cave has assesed by their publication in 2013 and the 22 phylum of the bacteria in addition to 5 phylum of the archaea have identified as capable of sulfur cycling (Güleçal and Temel; 2013, 2016a). At the other study in 2016, performed in Oylat Cave, the phyla, as proteobacteria dominated, other than composed by Actinobacteria, Acidobacteria and Nitrospirae has determined (Güleçal and Temel; 2016b).

2. MATERIALS AND METHODS

2.1 Site

Samples were collected from Insuyu cave located about 2 kilometers south of the village Yakaköy (previously Kurnaköy) at the 17. km of the highway laying from center towards to south, in Burdur Province, at the central of Turkey (Figure 2.1).

Figure 2.1 : Location of the Insuyu Cave.

(https://www.google.com.tr/maps/place, Reviewed on: November 23, 2016)

The cave mainly settle in the contact zone of three different formations which are Söbüdağ and Senirce Formations characterized by Upper Creteaseous Limestones and Gökçebağ Ophiolitic Melange. According to Erdogan et al. (2014), it is clear that the cave has formed in mainly Akdağ Limestone Units as relicts in Gökçebağ Ophiolitic Melange regarding to observations about non-karstified relicts of ophiolitic melange at the walls through the cave. Gökçebağ Ophiolitic Melange settled down the area at Upper Createase-Early Paleocene overlays the autocthonous Senirce and Söbüdağ Formations by structural unconformity. Also, all of three formations are covered by Plio-Quaternary alluvial sediments at the area extended towards to Southwest-West-Nortwest from the cave entrance. Besides, the area in which the cave has formed is sited at where the shear zone of small faults as the synthetic fault line of Burdur-Fethiye Fault and an antithetic fault line are dominant in whole of the basin. Hence, the effect of tectonism could be partly observed on the structure of the cave in addition to both Senirce and Söbüdağ Formations (Figure 2.2).

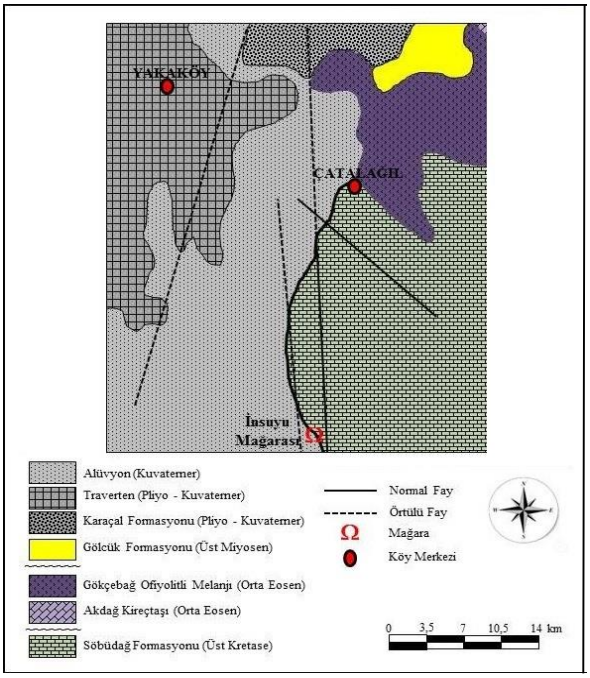


Figure 2.2 : Generalized geological map of the area. (Modified from: Erdogan et al., 2014)

According to our explorations, this ~10 km long cave formed by solution of the relict carbonate rocks of Gökçebağ Ophiolitic Melange have seven main galleries exhibiting stalactites, stalagmites, and columns within ten big underground lakes. It could be considered as two parts. The first part of the cave, which is operated as a touristic site, is comprised by a long passage about 250 meters long and an average width of 2 meters, a part in sponge-like characteristic within many small branches, a chamber and one main gallery with a lake. The second part of the cave is composed by six galleries connected by passageways and chambers with many lakes in a diverse array of sizes. The passages and chambers at the second part of the cave also do not have uniform size and shape. It has illuminated that the sponge-like form of the part at the first division also exits under the lakes of this part. While the floor of the first part is almost level, the floor at the second part is shaped with boulders and deposits of clay and silt which are settled on just top of the structure with this sponge-like characteristic (Figure 2.3).

The first gallery(A) located at the end of the touristic walkway is in NE-SW direction about 90 meters long and 25 meters wide as two parts. The first part is a low-ceiling chamber with a lake and the second part is another chamber next to the first one with the floor as a white powder-like sand plain beneath a sharp inclination constituted with boulders originated from collapsed

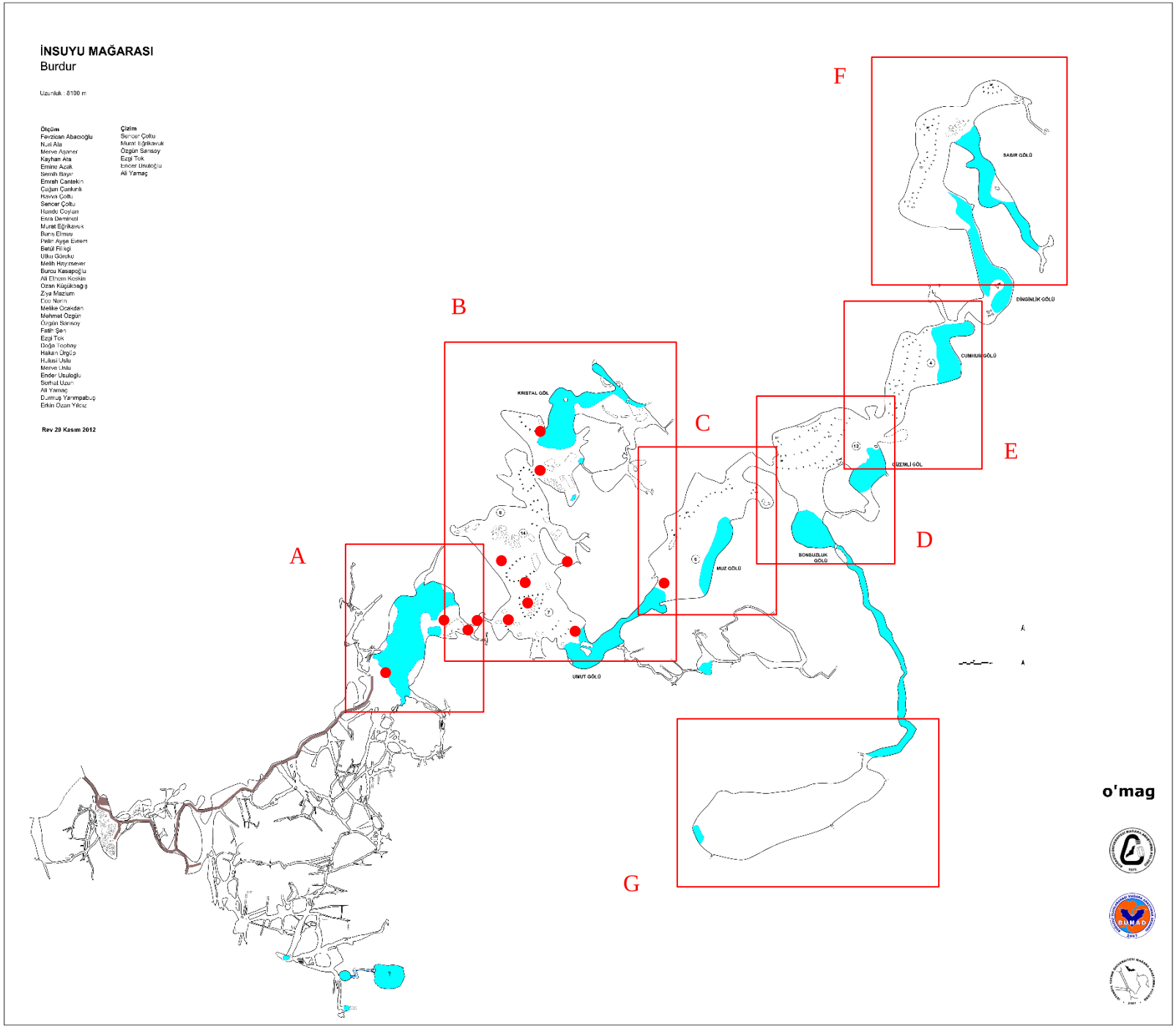


Figure 2.3 : The map of Insuyu Cave.

ceiling. The second chamber also has a small lake derived from underground water of basin, similarly the all of lakes in the cave (Figure 2.4a).

(a) **(b)**

Figure 2.4 : (a) Big Lake at the first gallery of Insuyu Cave. (b) The second gallery named Crystal Lake and needle-like crystals.

The second gallery(B) is in N - S direction within three chambers. The first chamber, coming right after a narrow passage connecting with the first gallery, is relatively smaller with about 60 meters length and 25 meters width with an average height of 20 meters. The foor is shaped as a hill composed of the boulders falling down. The hill sharply inclines towards three direction except the direction coming from the narrow passage. The second chamber extends after the sharp inclination at the northern part of the first chamber. It is a bigger chamber, about 70 meters long, 50 meters high with an average width of 40 meters. The most striking features of this chamber are $CaCO^3$ accumulations on ceilings and walls (Figure 2.4b). The third chamber follows the second one with a floor covered by boulders and partly flooded by an underground lake at Northeastern.

After the second gallery, a narrow passageway extends in NE - SW direction for about 90 meters. Its height is maximum 6 meters and it is about 10 meters wide. This passage is also flooded by underground waters as a lake and the direction of the flow is same as the direction of the passage (Figure 2.5).

Figure 2.5 : Passageway lake named Hope Lake, Insuyu Cave.

The third gallery(C) is in NE – SW direction. The ground is covered by deposits of silt and clay which are derived from the partly flooded underground lake according to the changes in the water level. It is about 140 meters long and 50 meters wide.

The fourth(D) and the fifth(E) galleries have the same characteristics with the third one except the fault which crosses over them. This fault is the best example observed in cave which represents the tectonism shaping the formations at the area. It is indicated on the middle of the ceiling at the fourth gallery while it is on the side wall at the fifth gallery.

Most of the six gallery(F) is occupied by a lake at two branches. The gallery extends 180 meter long in the direction N - S and has 10 to 30 meters in width. Here the height of the gallery is just 3 meters at its highest point. A sheet of small stalactites and a wide range size columns occupy the ceiling and the floor. The end of this gallery is the furthest point of cave from the enterance.

The last gallery(G), extending in E – W direction, 150 meters long, and about 40 meters wide follows the fourth gallery. Towards the end of this gallery, the direction changes to NE - SW. After a narrow passageway comes a small room and a lake.

As we have previously mentioned, the cave has formed in formations composed of fractured limestones, and the limestones in the report area are the most abundant rock in general. Most

of the limestone layers are thin to thick bedded, although some are horizontally laminated. Many of the layers are remarkably persistent, and some have been traced for kilometers. The limestones in this area were most probably formed by the chemical and mechanical deposition of carbonate in a humid environment.

2.2 Sampling and Microbiological Analysis

2.2.1 Sampling

The samples of bacteria and archaea colonies were collected from walls, floor and ceiling of the cave, in July 2015. Microbial communities habitating on different areas at the first and the second part of the cave were spotted regarding to the typical geological features as evidence

Figure 2.6 : Samples from different locations of Insuyu Cave.

of microbial activity. Within two sampling trip, the host-rock were sampled at 18 locations, the one at the touristic part and the rest of them at the second part, as 16 of them from the first trip and 2 additional ones from the second trip (Figure 2.7).

Within this area, 18 samples including the deposits of various minerals and the altered rock surfaces, each covering an area of approximately 10 cm^2, were taken by scraping off with a sterilized scalpel in 250-mL sterile polypropylene tubes and 50-mL falcon tubes, without

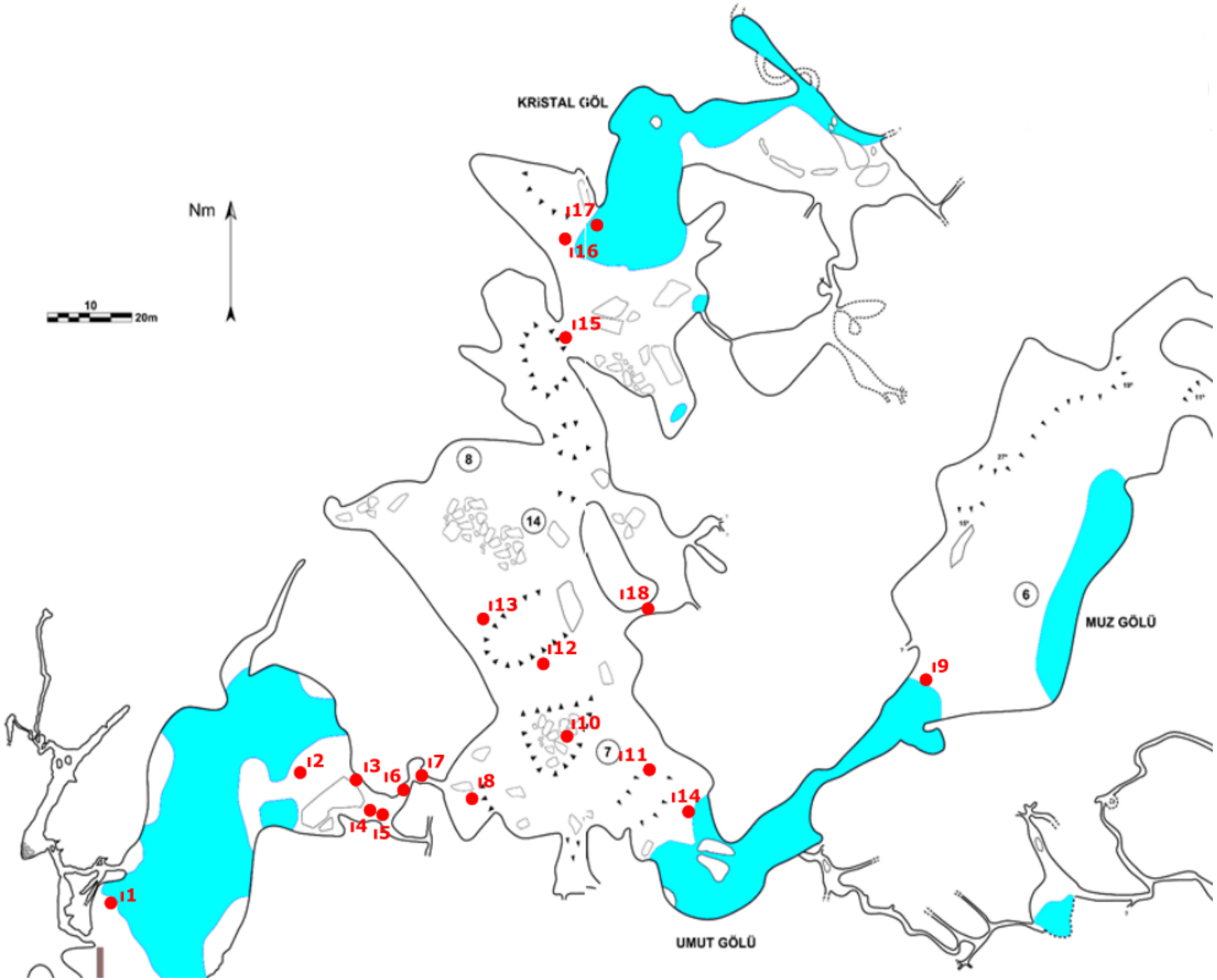


Figure 2.7 : The sampling locations on the detailed map of Insuyu Cave.

touching the supporting rocks. The samples were represented by white needle-like crystals, blackish-grey muddy depositions, reddish crusts and depositions with black velvet texture covering on white mud (Figure 2.6).

All sampling points have been photographed and marked on the cave map The photographs of sampling points have been given in Table 2.1, in addition to the photographs and short descriptions of the samples.

All samples were preserved in their original state without any additional fluid or chemical. Conductivity, pH and temperature were measured in situ during field sampling using portable instruments (ExStik2, Extech Instruments, NH 03063 U.S.A). Upon collection, the samples were transported in portable freezer with ice cassettes and stored at -20 ^{0}C in the laboratory.

Table 2.1 : Samples from different locations of Insuyu Cave. (To continue)

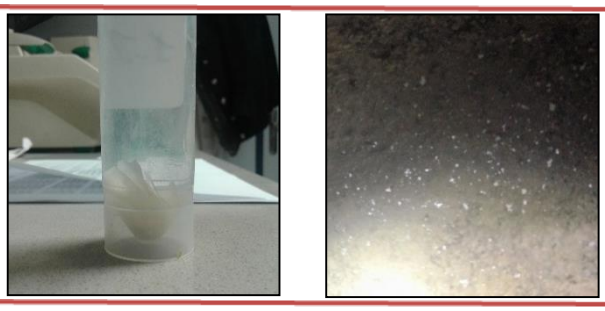
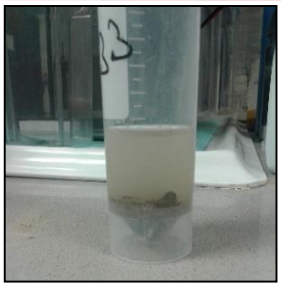
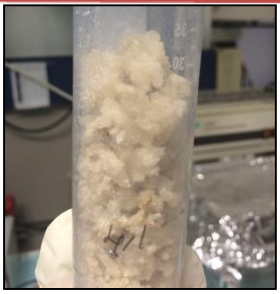
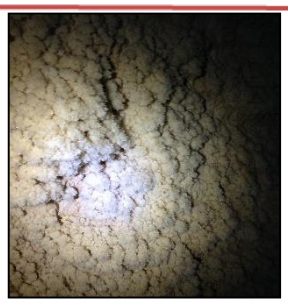
I1		Opaque white, carbonate folio from surface of Big Lake
I2		White powder-like carbonate sand from beach behind Big Lake
I3		Black mud from the rock surface, a meter above ground level
I4	 	White popcorn-like cyristals from the wall

Table 2.1 : Samples from different locations of Insuyu Cave. (To continue)

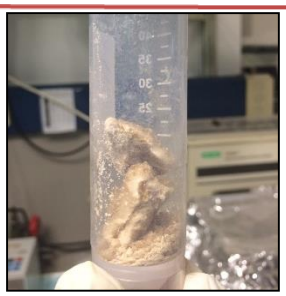
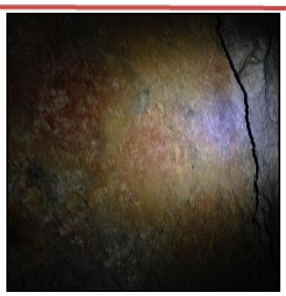
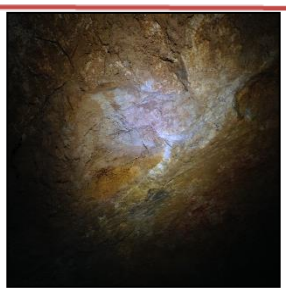
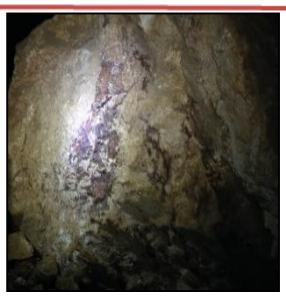
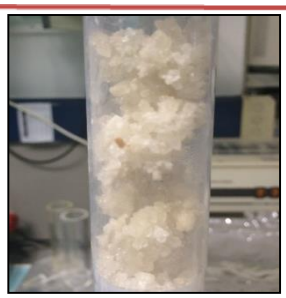
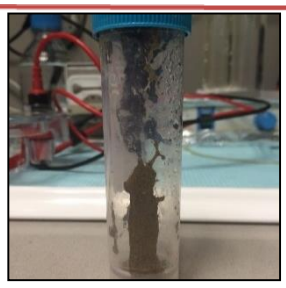
I5			White popcorn-like cyristals from the floor, just below the point I4
I6			Reddish mud from the wall
I7			Reddish crust from the wall
I8			Black crust from the wall
I9			White popcorn-like cyristals from the wall
I10			Black mud from the floor

Table 2.1 : Samples from different locations of Insuyu Cave. (To continue)

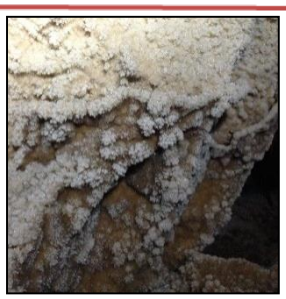
I11			White popcorn-like cyristals from the floor
I12			White needle-like cyristals from the wall
I13			Black mud layer under the white needle-like cyristals from the floor
I14			Deposition from the edge of Hope Lake
I15			Brown crust with the growing white needle-like cyristals from the wall
I16			Deposition under needle-like cyristals from the old edge of Cyristal Lake

Table 2.1 : Samples from different locations of Insuyu Cave. (To continue)

I17			Mud from the ground of Cyristal Lake
I18			Black velvet-like layer with blue and gray spots (colonies?) from the wall 50 cm above from the ground level

2.2.2 Microbiological analysis

2.2.2.1 Table of analysis, equipments, buffers, reagents and enzymes

The methods applied to each sample in the context of this study are outlined in Table 2.2.

Table 2.2 : The samples obtained from Insuyu Cave versus the analysis applied. (To continue)

	Extraction	***PCR***	***HRM***	***NGS***
I1	X	X		
I2	X			
I3	X	X	X	X
I4	X			
I5	X	X	X	
I6	X	X	X	X
I7	X	X	X	
I8	X	X	X	
I9	X			

I10	X	X	X	
I11	X	X	X	X
I12	X			
I13	X	X	X	X
I14	X			
I15	X	X		
I16	X	X	X	X
I17*	X		X	X
I18*	X		X	X

*Marked samples have taken only at the second trip.

The laboratory equipment used during this study is listed in Appendix A. The compounds and enzymes used during this study are listed in Appendix B.

2.2.2.2 DNA extraction

DNA were extracted from the samples by The PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., CA) with the modification on the producer's protocol. At the first experiments, the results from agarose gel electrophoresis represented that the possible inhibition during DNA extraction. Ca^{2}+ was determined as inhibition factor by XRF analysis and the alternating copolymer, poly-deoxyinosinic-deoxycytidylic acid sodium salt, was used to increase the yield of DNA extraction via blocking the reaction which Ca^{2}+ attaches to released bounds of one of the separated pairs of DNA molecules (Barton et all., 2007).

After the extraction of DNA, each sample analyzed on agarose gels. The gels were prepared using 1% (w/v) agarose in 1XTAE buffer containing 0.5 μg/mL ethidium bromide. 4μl of DNA samples were mixed with 1μl 6x loading dye. Electrophoresis was performed at 10V/cm and the gel was visualized under UV using gel imaging system (Gel Doc, BIORAD, US). Samples were stored at -20 ^{0}C until used.

2.2.2.3 Polymerase chain reaction (PCR) and cloning

11 of 18, which are mineralogical different from the others, were chosen for the experiment (Appendix C). Microbial communities were monitored by 16S rRNA gene based PCR (Polymerase Chain Reaction) methodology. Nested PCR approach was used to amplify microbial rDNAs. The samples were amplified by using Bacterial universal primers which are PA (5'AGAGTTTGATCCTGGCTCAG) - PH (5'AAGGAGGTGATCCAGCCGCA) at the first round and VFGC-VR at the second round, and Archaeal universal primers which are 7F-1384R at the first round and 344FGC-522R at the second round. Each PCR mixture was prepared in a final volume of 25 μL: 1 μL of template DNA, 0,5 μL of each primer (final concentration, 100 nM) and Taq Polimerease, 1 μL of dNTP, 19 μL of PCR-grade water, and 2,5 μm L of 1x Buffer with $MgCl^2$. PCR was then carried out with five minutes of initial denaturation at 94 ◦C, 30 cycles at 94◦C for 1 min, 55◦C for annealing with the duration one minute, and 72◦C for 1 min and 30 s, and then a final extension period of 8 min at 72◦C.
After, then, the PCR products were separated on a 1,5% agarose gel following the staining with ethidium bromide in 1X TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM 0.5 M ethylenediaminetetraacetic acid, pH 8.0). AGE was conducted for 30 minutes at 100 V cm-1 and the products were monitored by using trans-UV (BIORAD, USA).

2.2.2.4 High resolution melting curve (HRM) analysis

Subsequently, archaeal and bacterial diversities in cave sediments, were investigated to be able to compare with respect to their similarities by using HRM. Nested qPCR approach was used to amplify microbial rDNAs (Kolukirik et al. 2011). The first round qPCRs were carried out using Arch46f-Arch1017r and Bact8f-Bact1541r primer sets targeting archaeal and bacterial rRNA coding genes respectively. Arch344f-Univ522r and Bact342f-Bact534r primer sets were used for the second round PCRs. The following thermal cycling conditions were applied for all of the qPCRs: 3 mins at 95 °C; 40 cycles of 20 secs at 95 °C, 20 secs at 53 °C and 30 secs at 72 °C. Biospeedy™ HRM Master Mix (Bioeksen Ar- Ge Teknolojileri, Turkey) and Biorad CFX connect instrument was used for all reactions. The reactions contained 1.5mM MgCl2, 0.2mM dNTP mix, 1x Reaction Buffer, 0.1U Fast Start Proof Reading Recombinant Taq DNA Polymerase, 1x EvaGreen, 5ng/μL DNA template and 0.5μM of each primer. To ensure and detect whether if the expected product is amplified during q-PCR and for HRM analysis, melting curve analyses were applied between 60°C-95°C at a florescence reading rate of 0.1°C/acquisition. HRM profiles were obtained as described by Reja et al. (2010).

Microbial community profile dendrograms were obtained using Minitab 17 software based on the similarities between the HRM profiles. The correlations were evaluated using Pearson's method. Statistical significance was taken as $p < 0.05$. The first principal component (PC1) for the archaeal and bacterial HRM profiles have the eigenvalue 43.629 and 55.587 that accounts for 92.8% and 94.8% of the total variance, respectively. In other words, most of the HRM fingerprinting data structure was captured in PC1.

2.2.2.5 16S rRNA Metagenomic Sequencing

The protocol includes the primer pair sequences for the V3 and V4 region of the 16S rRNA that create a single amplicon of approximately 460 bp (Klindworth et al. 2013). The protocol also includes overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters. Illumina adapter overhang nucleotide sequences-16S rRNA specific sequences were 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWG CAG-3' for the forward primer and 5'-GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAG-GACTACHVGGGTATCTAATCC-3' for the reverse primer. The first PCR was performed using BiospeedyTM Proof Reading DNA Polymerase 2x Reaction Mix (Bioeksen Ltd Co., Turkey) and 200 nm of each primer. The following program was performed on Biorad CFX Connect Instrument (Bio-Rad Laboratories, U.S.A.): 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; 72°C for 5 minutes. The PCR product was run on an agarose gel to verify the size (~550 bp) and purified using BiospeedyTM PCR Product Purification Kit (Bioeksen Ltd. Co., Turkey).

The dual indices and Illumina sequencing adapters were attached to the purified first PCR products via the second PCR that was run using the Nextera XT Index Kit (Illumina Inc., USA) and the following program: 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; 72°C for 5 minutes. The PCR products were purified using BiospeedyTM PCR Product Purification Kit (Bioeksen Ltd. Co., Turkey). The final library was run on a Bioanalyzer DNA 1000 chip to verify the size (~630 bp). The final library was diluted using 10 mM Tris pH 8.5 to 4 nM and the 5 µl aliquots were mixed for pooling the libraries. In preparation for cluster generation and sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer (HT1), and then heat denatured before MiSeq sequencing. Illumina MiSeq v3 reagent kits were used for the runs. Each run included a minimum of 5% PhiX to serve as an internal control.

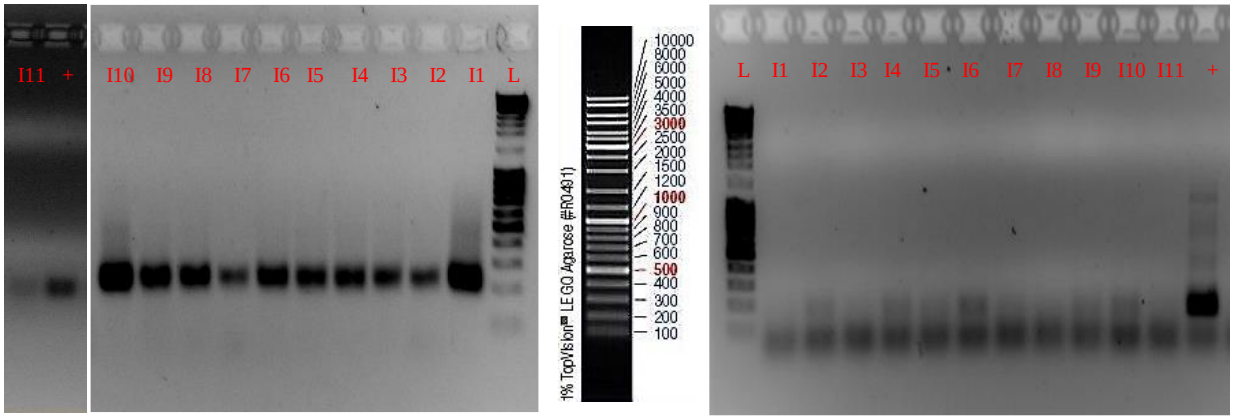
The raw sequence data (concatenated forward and reverse sequence reads) were cleaned, reduced and analyzed using Mothur Version 1.36.1. Firstly, the barcode and the primer sequences were trimmed and then unique sequences were identified. The trimmed unique sequences were aligned to the SILVA rRNA database sequences using blastn algorithm (Pruesse et al., 2007). Before this the SILVA database sequences were trimmed to include only the V3-V4 region. The overhangs at both ends were removed via filtering the sequences and the redundancy check was carried out. For further de-nosing, the sequences were pre-clustered. The chimeras were eliminated using the implanted code UCHIME (Edgar et al., 2011). The sequences were classified by using Bayesian classifier implanted in mother. The reference and taxonomy files were adopted from the SILVA database (Pruesse et al., 2007). After Operational Taxonomic Unit (OTU) picking and their taxonomic assignment using the SILVA rDNA database, the OTUs were binned in to phylotypes.

3. RESULTS AND DISCUSSION

3.1 Results

A total of 11 speleothem sample were investigated in this study. First, 16S rRNA genes were amplified to determine the presence of any archaeal and/or bacterial community in order to that there is inadequate amount of 16S rRNA genes to monitor the presence of colonies after extraction. As a result of this operation, it has confirmed that both bacterial and archaeal 16S rRNA genes are present on all samples (Figure 3.1).

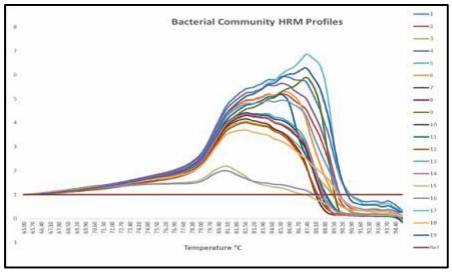
Then, the community profiles were investigated by HRM method. 2 of 11 samples, which were previously analysed by PCR, were excluded because of high similarity in physical features with other samples. However, 2 another samples taken at the second field trip were added to samples taken at the first trip and analysed in HRM. Finally, 11 sample were analysed by HRM method. Both the samples analysed by PCR and HRM were given at the table Appendix D. Regarding to the results of this experiment, microbial community profile were illustrated with the dendrograms graph based on the similarities between the HRM profiles of samples (Figure 3.2 a;b;c;d).



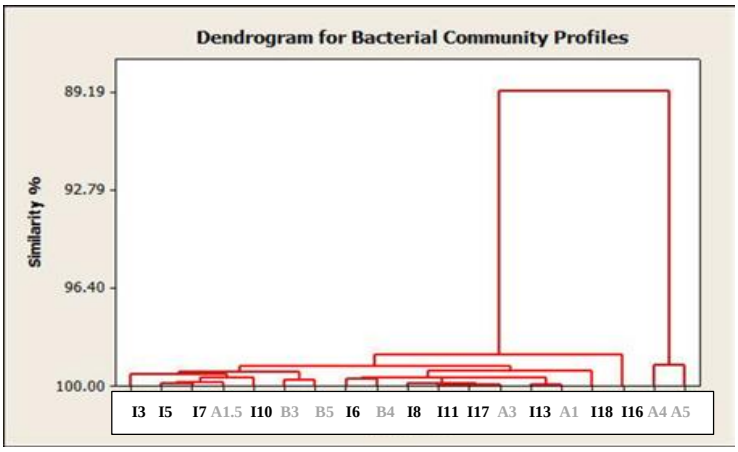
(a) **(b)**

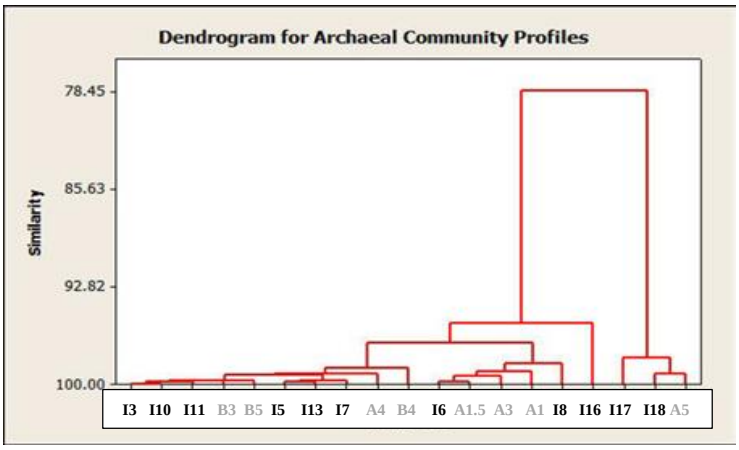
Figure 3.1 : Agarose gel electrophoresis (1,5% w/v) photos of 16S rRNA genes PCR results. (a) Eubacterial 16S rRNA genes PCR results of Insuyu Cave (Left: Lower part of the gel; Right: Upper part of the gel with modification on exposure of the image.) (b) Archeal 16S rRNA genes PCR results of Insuyu Cave.

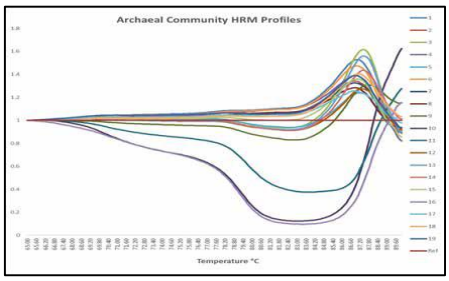
Two outliers seen at the eubacterial HRM profile graph are irrelevant with this study cause of belonging to the data set of another cave, but two of three outliers seen at the archaeal HRM profile graph are I17 and I18.



(a) Eubacterial HRM Profiles.
(b) Dendrograms graph of Eubacterial HRM profiles of Insuyu Cave.







(c) Archaeal HRM Profiles.
(d) Dendrograms graph of Archaeal HRM profiles of Insuyu Cave.

Figure 3.2 : The dendrograms graph based on the similarities between the HRM profiles of samples

According to this graph, 7 of 11, with moderate similarity to the others, were chosen for 16S rRNA metagenomic sequencing to represent each one of different communities in the cave ecosystem.

Subsequently, the chosen samples which are I3, I6, I11, I13, I16, I17 and I18 were analysed with next genaration sequencing method based on 16S rRNA metagenomics.

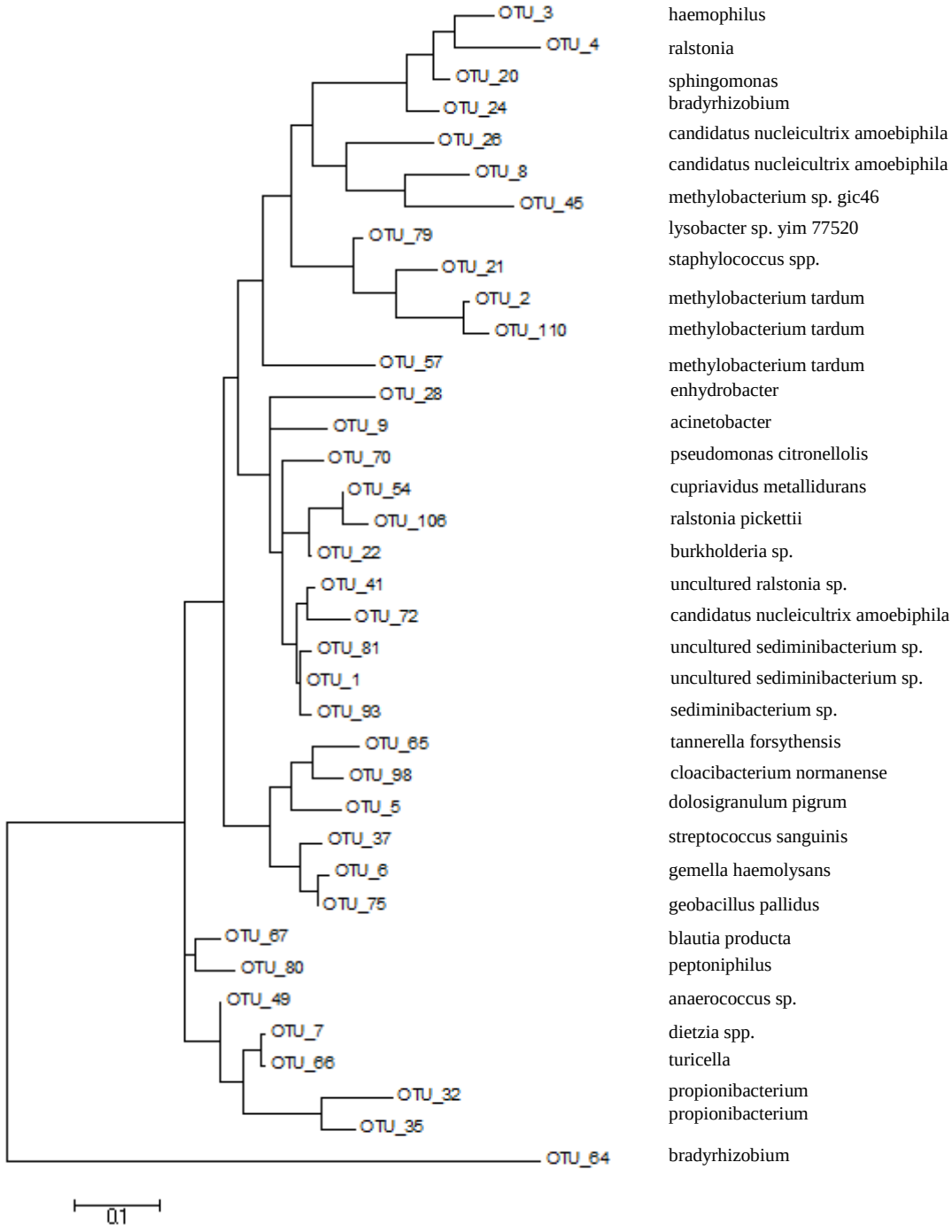


Figure 3.3 : The bacterial phylogenetic tree showing the relationships of bacterial 16S rRNA Metagenomic Sequence cloned from the studied samples to closely related sequences from the SILVA database.

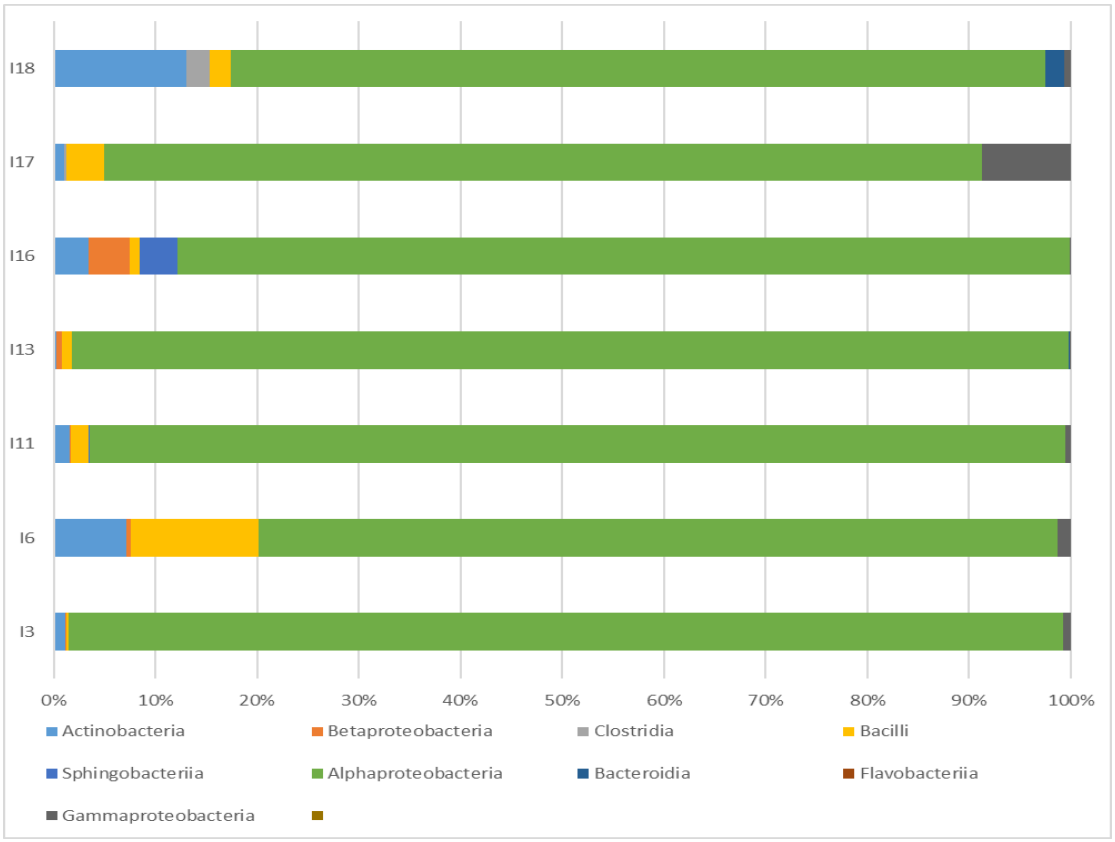


Figure 3.4 : Distribution of bacterial classes based on 16S rRNA gene clone libraries constructed from environmental DNA obtained from the Insuyu Cave microbial community.

Sequences were grouped with their most closely related class/genus using SILVA database. Mothur Version 1.36.1 genetic analysis software was used to produce the phylogenetic three of OTUs of the 16S clones from Insuyu Cave, after the elimination of chimeras with the implanted code UCHIME (Figure 3.3).

The bacterial phylogenetic tree were generated with the OTUs provided by Mothur based on evolutionary distance. A total of nine known bacterial class were found within the context of this research (Figure 3.4).

The most prevalent class is alpha proteobacteria with 89,23% of the total bacteria found grouping into four order, with the most abundant ones: rhizobiales (88,85%) and sphingomonadales (0,21%). The followings, ordered by abundancy, are actinobacteria (3,94%), bacilli (2,92%), gammaproteobacteria (1,69%) and betaproteobacteria (0,74%).

Also, a total of 14 genera from ten orders were identified in this study (Figure 3.5; Figure 3.6). The most dominant is methylobacterium with 88,83%, from the class of rhizobiales (88,85%).

The followings are propionibacterium (3,58%), dolosigranulum (1,69%), streptococcus (1,10%) and pseudomonas (1,13%).

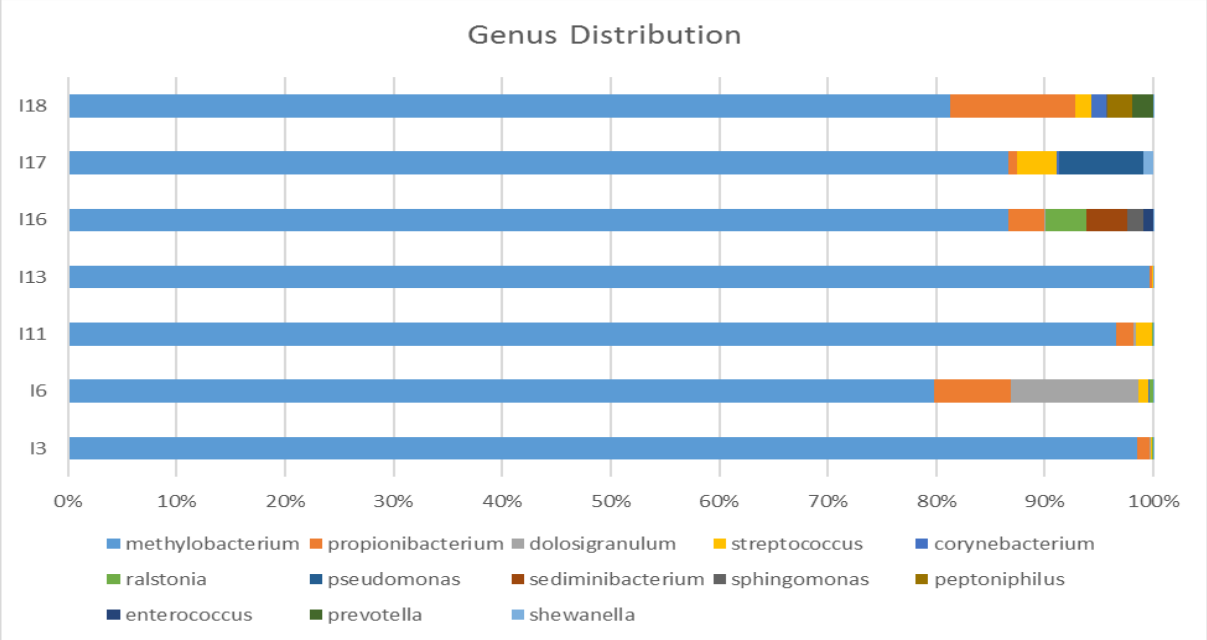
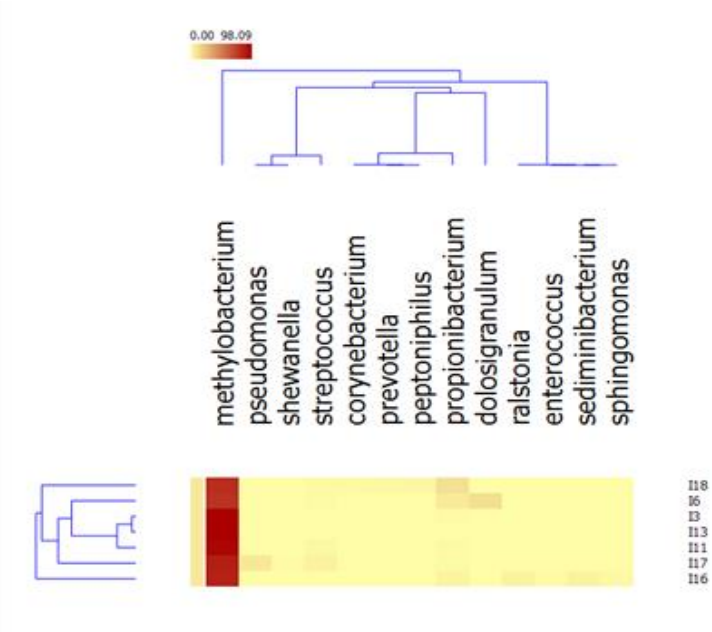


Figure 3.5 : Distribution of bacterial genuses based on 16S rRNA gene clone libraries constructed from environmental DNA obtained from the Insuyu Cave microbial community.



Horizontal: The genuses clustered regarding to their population density in Insuyu Cave ecosystem.

Vertical: The genuses clustered regarding to population density in the comparison of each one of the samples with the others.

Figure 3.6 : Heatmap analysis of bacterial diversity according to data of environmental DNA obtained from the Insuyu Cave microbial community.

As shown at the Figure 3.6, the samples I3, I13 and I11 are the most similar ones in the context of population density and distribution. Also, it is represented that the genus methylobacterium is intensively denser than the other genuses.

With 16S rRNA metagenomic sequencing, although detailed information on bacterial communities could be reached, the data could be obtained on archaeal communities are inadequate to comment.

3.2 Discussion

In this study, the samples which are I3, I6, I11, I13, I16, I17 and I18 were analysed with next generation sequencing method based on 16S rRNA metagenomics and the analysis showed that the microbial communities of the samples obtained from Insuyu Cave are mainly composed of alphaproteobacteria regarding to the bacterial 16S rRNA gene clone libraries. Many previous studies have also reported the microbial predominancy of alphaproteobacteria in addition to actinobacteria, gammaproteobacteria and betaproteobacteria (Barton et al., 2004; Schabereiter-Gurtner et al. 2002; Laiz et al., 2000).

As previously mentioned and shown at the Figure 3.6 in chapter 3.1, the samples I3, I11 and I13 are the most similar samples in the context of population density and distribution. The genera of propionibacterium, ralstonia and methylobacterium comprise to the bacterial flora of I3. The bacterial flora of I11 is composed by streptococcus and prevotella in addition to propionibacterium and methylobacterium, while the bacterial biodiversity of I13 is simply constituted of propionibacterium and methylobacterium.

Also, among the samples analyzed by NGS, the samples with the highest similarity in terms of mineralogy are I11 and I13. In addition to that the same cyristal formation can be observed at both of the points I11 and I13, both of the locations are sited at former lake areas. Furthermore, I3 is considerably close to the carbonate sand beach, which is also another old lake ground. On the other hand, there are no signs of mineralization at I6, a fossil passage, and at I17 where water activity is still present. However, it is unexpected that the microbial community of I18 shows such difference than the communities of I11 and I13 samples because of the point is sited at the small passageway connecting to the gallery which I13 was sampled from, and holding the same cyristal formations. Moreover, although it is composed of similar carbonate fibers, it is unlikely that I16 has a bacterial flora completely different from I11 and I13 except methylobacterium. These two situations will be investigated by mineralogical analyzes in future studies.

Barton et al.(2004) have documented by their research conducted in Fairy Cave, Glenwood Springs, Colorado that the most abundant group in the cave has identified as the members of the methylobacteria with the class of alfaproteobacteria, also responsible with Type I formaldehyde assimilation. The conclusions of Barton's study support our results representing that the genus methylobacterium is intensively denser than the other genuses.

They also have reported that the other significant group includes the members of the genus sphingomonas belonging the order spingomonodales. Members of this group are very well known with the capability of metabolize a diverse range of aromatic compounds (Kelly et al., 2004; Balkwill et al. 1997). Because of that, the members of this genus have named studies on oligotrophic ecosystems (Barton et al., 2004; Kelly et al., 2004). Kelly et al. (2004) have suggested that the "shower curtain biofilm", which is constituded from extracellular polymeric substance (EPS), is derived from the metabolic activity of the genus sphingomonas. The members of this genus were mainly determined on the sample I16 which is a speleothem in the shape of a layer constituting with needle-like calcite cyristals. Many studies have suggested that EPS has a great importance on the microbially induced calcification (Canaveras et al., 2001; Northup and Lavoie, 2001; Canaveras, 1999). Hence, it could be the same relation between our sample and sphingomonas activity.

The other class, actinobacteria (a.k.a Actinomycetes) are marked as the most abundant group of bacteria in the ceiling and walls of Altamira and Tito Bustillo Caves, Spain (Canaveras et al., 2001) Also, Canaveras et al. (2001) have described the speleothems which the colonies were isolated as needle-fiber calcite and/or aragonite crystals within random microstructural fabric. The sample named I18 which almost whole population of propionibacterium, the order of the class actinobacteria, colonizes on shows same crystal structures within the black velvet-like mat.

Besides, it is determined that the one of the most abundant populations in CSPC ferromanganese deposits is pseudomonas, from the class of gammaproteobacteria, by the research on the relevancy of Mn(II)-oxidizing bacteria with the ferromanganese deposits in caves of the Upper Tennessee River Basin (Carmichael et al., 2013). Similarly, the sample I17 which the huge part of pseudomonas population inhabitates on were taken cliff wall of the underground lake at Insuyu Cave. The sample has also characteristic feature which is black crust.

Further work may underway to elucidate the relaionship between the microbial diversity profile and mineralization processes by supporting the findings of this study with experimental

evidences of XRF, XRD and SEM-EDS electroscopy analysis in addition to measurement of ionic saturation index of cave water for anions and nitrogen species by using single column ion chromatography (IC) and for major cations, silica, and metals by using Inductively Coupled Plasma Emission Spectrometer (ICPES) or Inductively Coupled Plasma Emission Spectrometer (ICPMS) (Gulecal-Pektas and Temel, 2016; .Baskar et all., 2011; Rogers et all., 1998). Additionally, culture based methods may also considered as a supportive technique by using various selective media such as B-4 media and Mn agar for calsium carbonate and hydromagnesite precipitation (Baskar et all., 2011; Banks et all., 2010; Canaveras et all., 1999) or Boston's Basal Salts for ferromanganese precipitation (Spilde et all., 2005).

On the other hand, no archaeal species has been detected at the result of NGS analysis while some archaeal nucleic acid had been marked as a result of PCR analysis. The reasons of this situation has been investigated in the section of discussion. With no certainty, there are some possible explanations existing. Due to the scarce nucleic acid concentrations, to obtain consistency between the results as confirming the existence of microorganisms has been a competitive situation throught the research. Even all conditions have obtained as similar, random errors had intensive effect on the results of extraction and PCR procedures. Moreover, the factors inhibitating the extraction reagents yields are also present such as Ca^{2+} anions cause of the sedimental origin of the samples.

There might be an error occurring at the second extraction or an inhibitation at the second PCR processes approached without poly-deoxyinosinic-deoxycytidylic acid sodium salt as differently from the first PCR analysis performed as a part of NGS analysis. Hence, depending on these reasons, it is a possibility not to observed archaeal nucleic acids in very low concentrations, which are also naturally in low concentrations compearing to bacterial ones. Within the further studies, the reasons and solutions of this situation will be investigated.

As a primary suggestion, the PO^4 treatment may be discussed in case of that to increase the amount of poly-deoxyinosinic-deoxycytidylic acid sodium salt treated is not posssible because of that the excessive load of poly-deoxyinosinic-deoxycytidylic acid sodium salt can also cause a negative effect on the extraction yield.

Lever et all. (2015) have suggested the PO^4 treatment and reported increase in efficiency especially on archaeal extraction as a result of the study with the aim to develope a separation method with higher yield for DNA pools from diverse environmental samples. It has revealed that further addition than 450 μmol g−1 sample of PO^4 increases archaeal copy numbers by a factor of five while there is also little changes in archaeal gene copies in case of the treatment

from 45 to 150 μmol g−1 sample at the research conducted with the oligotrophic sediment sampled at Subglacial Lake Whillans. In the study, the researchers have also compared to the kits that we used and their extraction protocol and stated that approximately one order of magnitude higher yield has been reached.

Within the context of this study, we describe the isolation and phlogenetic identification procedure of archaea and bacteria species derived from speleothems collected Insuyu cave, a karstic cave located near the center of Burdur Province, Turkey.

Finally, we discuss the possible connections between the communities of bacteria isolated from different locations in cave and the environment where they settled on. Also, the difficulties at the monitoring of the microbial community isolated from environmental samples and the inconsistency between the results of analysis on archaeal communities have been discussed and the probable solution of the situation has been suggested.

REFERENCES

Amann R.I., Ludwig W. & Schleifer K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev*, 59, 143–169.

Banks E.D., Taylor N.M., Gulley J., Lubbers B.R., Giarrizo J.G. & Bullen H.A. (2010). Bacterial Calcium Carbonate Precipitation in Cave Environments: A Function of Calcium Homeostasis. *Geomicrobiology Journal*, 27, 444-454.

Barıs O., Yanmıs D., Karadayı M., Gulluce M. & Sahin F. (2012). Mağara Kaynaklı Bakterilerin moleküler Tiplendirilmesinde ARDRA'nın (Amplified Ribosomal DNA Restriction Analysis) Kullanımı. *2.Ulusal Moleküler Biyoloji ve Biyoteknoloji Kongresi Antalya/Türkiye.*

Barıs O., Yanmıs D., Gulluce M., Karadayı M.& Sahin F. (2012). Oligotrofik Mağaralarda Bakterilerin Zaferi. *21. Biyoloji Kongresi İzmir.*

Baris O., Gulluce M., Adiguzel A., KaradayI M. & Sahin F. (2010). Mağara bakterileri ve oligotrofik. *V. Speleoloji Sempozyumu, İstanbul.*

Barıs O., Gulluce M., Ozkan H., Adıguzel A., Orhan F. & Sahin F. (2010). Elmalı (Pazaryolu-Erzurum) Mağaralarında Kalsit Oluşumuna (CaCO3) Katılan Bakterilerin Belirlenmesi. *20. Ulusal Biyoloji Kongresi, Denizli.*

Barıs O., Gulluce M. & Sahin F. (2010). Ezurum Karsit Mağaralarında Bakteriyal Biyomineralizasyon. *20. Ulusal Biyoloji Kongresi, Denizli.*

Baris O., Sahin F., Ozkan H., Adiguzel A. & Gulluce M. (2008). Yıldızkaya (Olur/Erzurum) mağarasında damlataşlarının oluşumunda etkili bakterilerin araştırılması. *4.Ulusal Biyomühendislik Kongresi, İzmir.*

Barış O. & Gulluce M. (2008). Yıldızkaya (Kivi) Mağarası Damlataşlarında Bakteri Florasının Araştırılması. *IV. Speleoloji Sempozyumu, Ankara.*

Baris O., Gulluce M., Sahin F., Ozkan H., Orhan F. & Ozbek T. (2008). Mağara Sistemlerinde Kalsit (CaCO3) Oluşumu ve Bakterilerin Etkisi. *19. Ulusal Biyoloji Kongresi, Trabzon.*

Barton H.A. & Northup D.E. (2007). Geomicrobiology in cave environments: past, current and future perspectives. *J Cave Karst Stud.*, 69, 163–178.

Barton H.A., Taylor N.M., Kreate M.P., Springer A.C., Oehrle S.A. & Bertog J.N. (2007). The Impact of Host Rock Geochemistry on Bacterial Community Structure in Oligotrophic Cave Environments. *International Journal of Speleology,* 36 (2), 93-104.

Barton H.A., Taylor M.R. & Pace N.R. (2004). Molecular Phylogenetic Analysis of a Bacterial Community in an Oligotrophic Cave Environment. *Geomicrobiology Journal*, 21, 11–20.

Baskar S., Baskar R. & Routh J. (2014). Speleothems from Sahastradhara Caves in Siwalik Himalaya, India: Possible Biogenic Inputs, *Geomicrobiology Journal*, 31(8), 664-681.

Baskar S., Baskar R. & Routh J. (2011). Biogenic Evidences of Moonmilk Deposition in the Mawmluh Cave, Meghalaya, India. *Geomicrobiology Journal, 28*, 14.

Baskar S., Baskar R. & Barton H.A. (2009). Cave geomicrobiology in the Indian context *Current Science, 97*(5), 4.

Boston P.J. (2000). Life below and life "Out There." *Geotimes*, 45, 14–17.

Boston P.J., Spilde M.H., Northup D.E., Melim L.A., Soroka D.S., Kleina L.G., Lavoie K.H., Hose L.D., Mallory L.M., Dahm C.N., Crossey L.J. & Schelble R.T. (2001). Cave biosignature suites: microbes, minerals, and Mars. *Astrobiology*, 1, 25–55.

Cacchio P., Contento R., Ercole C., Cappuccio G., Martinez M.P. & Lepidi A. (2004). Involvement of microorganisms in the formation of carbonate speleothems in the Cervo Cave (L'Aquila-Italy). *Geomicrobiology Journal*, 21, 497–509.

Canaveras J.C., Sanchez-Moral S., Soler V. & Saiz-Jimenez C. (2001). Microorganisms and Microbially Induced Fabrics in Cave Walls. *Geomicrobiology Journal*, 18, 223–240.

Canaveras J.C., Hoyos M., Sanchez-Moral S., Sanz-Rubio E., Bedoya J., Soler V., Groth I., Schumann P., Gonzalez I., Laiz L. & Saiz-Jimenez C. (1999). Microbial communities associated with Hydromagnesite and needle-fiber Aragonite deposits in a karstic cave (Altamira, Northern Spain). *Geomicrobiology Journal*, 16, 9–25.

Canganella F., Bianconi G., Kato C. & Gonzalez J. (2007). Microbial ecology of submerged marine caves and holes characterised by high levels of hydrogen sulphide. *Rev Environ Sci Biotechnol, 6*, 10.

Carmichael M.J., Carmichael S.K., Santelli C.M., Strom A. & Brauer L. S. (2013). Mn(II)-oxidizing Bacteria are abundant and environmentally relevant members of ferromanganese deposits in caves of the Upper Tennessee River Basin. *Geomicrobiology Journal*, 30, 779–800.

Cunningham K.I., Northup D.E., Pollastro R.M., Wright W.G. & LaRock E.J. (1995). Bacteria, fungi and biokarst in Lechuguilla Cave, Carlsbad Caverns National Park, New Mexico. *Environmental Geology, 25*, 9.

Engel A.S., Porter M.L., Stern L.A., Quinlan S. & Bennett P.C. (2004). Bacterial diversity and ecosystem function of filamentous microbial mats from aphotic (cave) sulfidic springs dominated by chemolithoautotrophic "Epsilonproteobacteria." *FEMS Microbiol Ecol*., 51, 31–53.

Ettema, T.J.G. and Andersson S.G.E. (2009, 11 March) The α-proteobacteria: the Darwin finches of the bacterial world. Rewieved on: November 21, 2016. http://rsbl.royalsocietypublishing.org

Ford, D.C. & Williams P.W. (2007). Karst Hydrogeology and Geomorphology. Wiley, Chichester.

Gatenby, J. B. (1960). The New Zealand Glow-Worm. *Journal of the Biological Society, 2*(8), 7.

Gillieson D. (1996). Caves: processes, development, and management. Oxford: Blackwell Publishers Ltd., 324 p.

Gulecal-Pektas Y. & Temel M. (2016). A Window to the Subsurface: Microbial diversity in hot springs of a sulfidic cave (Kaklik, Turkey). *Geomicrobiology Journal*, 0 (0), 1–11.

Gulecal-Pektas Y. (2016). Bacterial diversity and composition in Oylat Cave (Turkey) with combined Sanger/Pyrosequencing approach. *Polish Journal of Microbiology*, 65(1), 69–75.

Hose L.D., Palmer A.N., Palmer M.V., Northup D.E., Boston P.J. & Duchene H.R. (2000). Microbiology and geochemistry in a hydrogen-sulphide-rich karst environment. *Chem Geol*, 169, 399–423.

Hill C.A. & Forti P. (1997). Cave minerals of the world, 2nd ed. Huntsville, AL: National Speleological Society. 463 p.

Hill C.A. & Forti P. (1995). The classification of cave minerals and speleothems. *International Journal of Speleology*, 24, 6.

Hill C.A. (1990). Sulfuric acid speleogenesis of Carlsbad Cavern and its relationship to hydrocarbons, Delaware Basin, New Mexico and Texas. *Am Assoc Pet Geol Bull* ,74, 1685–1694.

Høeg O.A. (1946). Cyanophyceae and bacteria in calcareous sediments in the interior of limestone caves in Nord-Rana, Norway. *Nytt Magazin for Naturvidenskapene*, 85, 99–104.

Jones, B. (2001). Microbial Activity in Caves—A Geological Perspective. *Geomicrobiology Journal, 18*, 13.

Kelley S.T, Theisen U., Angenent L.T., St. Amand A. & Pace N.R. (2004). Molecular Analysis of Shower Curtain Biofilm Microbes. *Appl Environ Microbiol*, 70(7), 4187-4192.

Klimchouk A. (1994). Speleogenesis in gypsum and geomicrobiological processes in the Miocene sequence of the pre-Carpathian Region. In: Sasowsky I.D., Palmer M.V. (editors). Breakthroughs in karst geomicrobiology and redox geochemistry: abstracts and field-trip guide for the symposium, (p. 40–42). Colorado Springs, Colorado: Spec Pub 1. Charles Town, WV: KarstWaters Institute, Inc.

Klimchouk A. (2009). Morphogenesis of hypogenic caves. *Geomorphology,* 106, 100–117.

Kumeresan D., Wischer D., Stephenson J., Hillebrand-Voiculescu A. & Murrell J.C. (2014). Micrbiology of Movile Cave—A Chemolithoautotrophic Ecosystem. *Geomicrobiology Journal,* 31, 8.

Laiz, L., Groth, I., Schumann, P., Zezza, F., Felske, A., Hermosin, B. & Saiz-Jimenez, C. (2000). Microbiology of the stalactites from Grotta dei Cervi, Porto Badisco, *Italy. Int Microbiol*, 3, 25–30.

Lever M.A., Torti A., Eickenbusch P., Michaud A.B., Šantl-Temkiv T., Jørgensen B.B. (2015). A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. *Frontiers in Microbiology,* 6, 476.

Lozano R.P. & Rossi C. (2012). Exceptional preservation of Mn-oxidizing microbes in cave stromatolites (El Soplao, Spain). *Sedimentary Geology*, 255-256, 42–55.

Macalady J.L., Lyon E.H., Koffman B., Albertson L.K., Meyer K., Galdenzi S., Mariani S. (2006). Dominant microbial populations in limestone-corroding stream biofilms, Frasassi Cave System, *Italy. Appl Environ Microbiol,* 72, 5596–5609.

Marshall Hathaway J.J., Garcia M.G., Balasch M.M., Spilde M.N., Stone F.D., De Lurdes M., Dapkevicius N. E., Amorim I.R., Gabriel R., Borges P.A.V & Northup D.E. (2014). Comparison of Bacterial Diversity in Azorean and Hawai'ian Lava Cave Microbial Mats. *Geomicrobiology Journal, 31*(3), 16.

Marshall Hathaway J.J., Sinsabaugh R.L., De Lurdes M., Dapkevicius N. E., Northup D.E. (2014). Diversity of Ammonia Oxidation (amoA) and Nitrogen Fixation (nifH) Genes in Lava Caves of Terceira, Azores, Portugal. *Geomicrobiology Journal,* 31, 15.

Melim L.A., Shinglman K.M., Boston P.J., Northup D.E., Spilde M.N. & Queen JM. (2001). Evidence for microbial involvement in pool finger precipitation, Hidden Cave, New Mexico. *Geomicrobiology Journal,* 18, 311–329.

Northup D.E. & Lavoie K.H. (2001). Geomicrobiology of Caves: A Review. *Geomicrobiology Journal, 18*, 24.

Peck, S.B. (1986). Bacterial deposition of iron and manganese oxides in North American caves: *Bulletin of the National Speleological Society*, 48, 26–30.

Pacton M., Breitenbach S.F.M., Lechleitner F. A., Vaks A., Rollion-Bard C., Gutareva O.S., Osinzev A. V. & Vasconcelos C. (2013). The role of microorganisms on the formation of a stalactite in Botovskaya Cave, Siberia – palaeoenvironmental implications. *Biogeosciences Discuss*., 10, 6563–6603.

Pasic L., Kovce B., Sket B. & Herzog-Velikonja B. (2010). Diversity of microbial communities colonizing the walls of a Karstic cave in Slovenia. *FEMS Microbiol Ecol,* 71, 11.

Rogers J.R., Bennett P.C. & Choi W.J. (1998). Feldspars as a source of nutrients for microorganisms. *American Mineralogist*, 83, 1532–1540.

Rusznyák A., Akob D.M., Nietzsche S., Eusterhues K., Totsche K.U., Neu T.R., Frosch T., Popp J., Keiner R., Geletneky J., Katzschmann L., Schulze E.D. & Küsela K. (2012). Calcite Biomineralization by Bacterial Isolates from the Recently Discovered Pristine Karstic Herrenberg *Cave. Appl. Environ. Microbiol.*, 78(4), 1157.

Sanchez-Moral S., Portillo M.C., Janices I., Cuezva S., Fernandez-Cortes A., Canaveras J.C. & Gonzalez J.M. (2012). The role of microorganisms in the formation of calcitic moonmilk deposits and speleothems in Altamira Cave. *Geomorphology*, 139–140, 285–292.

Sarbu S.M. & Lascu C. (1997). Condensation corrosion in Movile Cave, Romania. *J. Cave Karst Studies*, 59, 99–102.

Schabereiter-Gurtner C., Saiz-Jimenez C., Piñar G., Lubitz W. & RöllekE S. (2002) Phylogenetic 16S rRNA analysis reveals the presence of complex and partly unknown bacterial communities in Tito Bustillo cave, Spain, and on its Palaeolithic paintings. *Environmental Microbiology*, 4(7), 392–400.

Spilde M.H., Northup. D. E.; Boston P.J., Schelble R.T, Dano K.E., Crossey L.J. & Dahm C.N. (2005). Geomicrobiology of Cave Ferromanganese Deposits: A Field and Laboratory Investigation. *Geomicrobiology Journal, 22*, 18.

Tomczyk-Zak K. & Zielenkiewicz U. (2015). Microbial Diversity in Caves. *Geomicrobiology Journal*, 18.

Winogradsky S. (1949). Microbiologie du sol, problemes et methodes. Paris: Masson, 861 p.

APPENDICES

APPENDIX A: Laboratory Equipment
APPENDIX B: Chemicals

APPENDIX A

Laboratory Equipment

Pipettes Eppendorf 2.5 µl, 10 µl, 20 µl, 100 µl, 1000 µl

Centrifuges Sigma 1-14

PCR Thermocycler BIORAD C1000 thermal cycler

Electrophoresis system BIORAD mini sub cell GT

Gel documentation system BIORAD GELDOC

Vortex Heidolph reax top

Autoclave TOMY SX-700E

Power supply BIORAD power pac 300

Refrigerators Whirlpool +4^{o}C, 20^{o}C, Vestel -20^{o}C; Haier -80^{o}C

Laminar flow Faster BH-EN 2003

Microwave oven Vestel MD17

APPENDIX B

Chemicals

Urea

Ethyl alcohol absolute SIGMA-ALDRICH

Tris-Acetate-EDTA molecular biology reagent SIGMA-ALDRICH

taq polymerase SIGMA-ALDRICH

Primers IONTEK

Ethidium bromide

O'GeneRuler DNA Ladder Mix THERMO SCIENTIFIC

6X Orange DNA Loading Dye THERMO SCIENTIFIC

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Educational Information

Master Degree Istanbul Technical University, Eurasia Earth Sciences Institute 2014-Still

Bachelor's Degree Istanbul Technical University, Food Engineering 2009-2014

Symposium and Congress / Publications

- Hypogea 2015, International Congress of Speleology in Artificial Cavities

  Water tunnels of Güvercinlik Valley (Cappadocia, Turkey). *Gilli Eric, Yamaç Ali, Tok Ezgi*
  Dovecotes and cave dwellings of Gesi - Kayseri (Turkey). *Tok Ezgi, Yamaç Ali*
  Underground cities of Kayseri (Turkey). *Yamaç Ali, Tok Ezgi, Filikci Betul*
- EGU General Assembly 2016

  The Primary Results of Analyses on The Archaeal and Bacterial Diversity of Active Cave Environments Settled in Limestones at Southern Turkey. *Tok Ezgi, Ahata Büşra, Kurt Halil, Akarsubaşı Alper T.*
- TURQUA 2017, Quaternary Symposium of Turkey

  Mağaralarda Arkeal ve Bakteriyel Çeşitliliğin İncelenmesine Bir Örnek: İnsuyu Mağarası. *Tok Ezgi, Ahata Büşra, Kurt Halil, Akarsubaşı Alper T.*
  (An Experimental investigation on the biodiversity profile of archaea and bacteria in a cave ecosystem: Insuyu Cave)

Social activity

- Istanbul Technical University Cave Exploration Club, 2009-2013

  Accounting Officer 2010-2011; President 2011-2012
- Istanbul Technical University Volunteerism Club, Another chance project, 2009-2011

  Lecturer 2009-2011
  (This is the project for the high school students in bad financial situation to prepare them to collage entrance exams)
- OBRUK Cave Exploration Group, 2011- Still
  Editor of OBRUK (Vertical publication- Caving Journal), Coordinator of Field Trips

Since 2009, at least 3 weeks in every year, I've achieved many field trips and cave expeditions at West, Middle and East Taurids; Diyarbakır, Urfa, Mardin, Konya and Kayseri Province and Küre Mountains National Park in Turkey.